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8181 Fannin, Houston, TX 77054 (US). WILSON, Deborah [US/US]; 11022 Silkwood, Houston, TX 77031 (US).

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(74) Agent: SHISHIMA, Gina, N.; Fulbright & Jaworski, L.L.P., Suite 2400, 600 Congress Avenue, Austin, TX 78701 (US).

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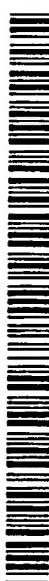
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(71) Applicants (*for all designated States except US*): INTROGEN THERAPEUTICS, INC. [US/US]; Suite 1850, 301 Congress Avenue, Austin, TX 78701 (US). BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th St., Austin, TX 78701 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): RAMESH, Rajagopal [—/US]; 5400 Braesvalley No. 96, Houston, TX 77096 (US). ROTH, Jack, A. [US/US]; 6516 Brompton, Houston, TX 77005 (US). SAEKI, Tomoyuki [—/US];



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(54) Title: METHODS AND COMPOSITIONS FOR NON-VIRAL GENE THERAPY FOR TREATMENT OF HYPERPROLIFERATIVE DISEASES

(57) Abstract: The present invention relates to non-viral gene therapy methods and compositions for treatment of hyperproliferative disease in humans. More specifically, the invention is directed, in one embodiment, to lipid formulations which form stable liposome structures, capable of efficient *in vivo* nucleic acid transfer. In other embodiments, methods and compositions are directed to liposome transfer of anti-proliferative nucleic acids, wherein the transfer of the nucleic acids is cell specific via cell specific targeting moieties. The present invention, thus provides non-viral, liposome compositions and methods of gene transfer particularly useful for targeting and treating hyperproliferative disease.

METHODS AND COMPOSITIONS FOR NON-VIRAL GENE THERAPY FOR TREATMENT OF HYPERPROLIFERATIVE DISEASES

BACKGROUND OF THE INVENTION

The present application claims the benefit of U.S. Provisional Application Serial Number 60/124,388 filed on May 24, 1999. The entire text of the above-referenced
5 disclosure is herein incorporated by reference.

A. Field of the Invention

The present invention relates generally to the fields of oncology and gene therapy. More particularly, it concerns the formulation of lipid based pharmaceuticals. In specific 10 embodiments, it relates to lipid based non-viral formulations related to gene delivery for treating hyperproliferative diseases.

B. Description of Related Art**1. Gene Therapy**

15 Gene therapy is an emerging field in biomedical research with a focus on the treatment of disease by the introduction of therapeutic recombinant nucleic acids into somatic cells of patients. Various clinical trials using gene therapies have been initiated and include the gene therapy of various cancers, AIDS, cystic fibrosis, adenosine deaminase deficiency, cardiovascular disease, Gaucher's disease, rheumatoid arthritis, 20 and others. Currently, the preferred vehicle for the delivery of gene therapy agents is the adenovirus. Advantages in using adenovirus as a gene therapy agent are high transduction efficiency, infection of non-dividing cells, easy manipulation of its genome, and low probability of non-homologous recombination with the host genome. Adenoviral gene therapy strategies, however, suffer from many inherent problems 25 including the potential for patient immune response, a possible inability to repeat administration of viral vectors, a difficulty in generating high viral titers, and the potential of infectious virus production. Non-viral delivery systems provide an alternative gene therapy vehicle that is devoid of one or more of these problems.

2. Lipid-Based Gene Transfer

Lipid-based non-viral formulations provide an alternative to adenoviral gene therapies. Although many cell culture studies have documented lipid-based non-viral gene transfer, systemic gene delivery via lipid-based formulations has been limited. A major limitation of non-viral lipid-based gene delivery is the toxicity of the cationic lipids that comprise the non-viral delivery vehicle. The *in vivo* toxicity of liposomes partially explains the discrepancy between *in vitro* and *in vivo* gene transfer results. Another factor contributing to this contradictory data is the difference in liposome stability in the presence and absence of serum proteins. The interaction between liposomes and serum proteins has a dramatic impact on the stability characteristics of liposomes (Yang and Huang, 1997). Cationic liposomes attract and bind negatively charged serum proteins. Liposomes coated by serum proteins are either dissolved or taken up by macrophages leading to their removal from circulation. Current *in vivo* liposomal delivery methods use aerosolization, subcutaneous, intradermal, intratumoral, or intracranial injection to avoid the toxicity and stability problems associated with cationic lipids in the circulation. The interaction of liposomes and plasma proteins is largely responsible for the disparity between the efficiency of *in vitro* (Felgner *et al.*, 1987) and *in vivo* gene transfer (Zhu *et al.*, 1993; Philip *et al.*, 1993; Solodin *et al.*, 1995; Liu *et al.*, 1995; Thierry *et al.*, 1995; Tsukamoto *et al.*, 1995; Aksentijevich *et al.*, 1996).

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Recent advances in liposome formulations have improved the efficiency of gene transfer *in vivo* (Templeton *et al.* 1997; WO 98/07408, incorporated herein by reference). A novel liposomal formulation composed of an equimolar ratio of 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP) and cholesterol significantly enhances systemic *in vivo* gene transfer, approximately 150 fold. The DOTAP:cholesterol lipid formulation is said to form a unique structure termed a "sandwich liposome." This formulation is reported to "sandwich" DNA between an invaginated bilayer or "vase" structure. Beneficial characteristics of these liposomes include a positive to negative charge or ρ , colloidal stabilization by cholesterol, two-dimensional DNA packing and increased serum stability.

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The production of lipid formulations often is accomplished by sonication or serial extrusion of liposomal mixtures after (I) reverse phase evaporation (II) dehydration-rehydration (III) detergent dialysis and (IV) thin film hydration. Once manufactured, 5 lipid structures can be used to encapsulate compounds that are toxic (chemotherapeutics) or labile (nucleic acids) when in circulation. Liposomal encapsulation has resulted in a lower toxicity and a longer serum half-life for such compounds (Gabizon *et al.*, 1990). Numerous disease treatments are using lipid based gene transfer strategies to enhance conventional or establish novel therapies, in particular therapies for treating 10 hyperproliferative diseases.

3. Cancer

Normal tissue homeostasis is a highly regulated process of cell proliferation and cell death. An imbalance of either cell proliferation or cell death can develop into a 15 cancerous state (Solyanik *et al.*, 1995; Stokke *et al.*, 1997; Mumby and Walter, 1991; Natoli *et al.*, 1998; Magi-Galluzzi *et al.*, 1998). For example, cervical, kidney, lung, pancreatic, colorectal and brain cancer are just a few examples of the many cancers that can result (Erlandsson, 1998; Kolmel, 1998; Mangray and King, 1998; Gertig and Hunter, 1997; Mougin *et al.*, 1998). In fact, the occurrence of cancer is so high that over 20 500,000 deaths per year are attributed to cancer in the United States alone.

The maintenance of cell proliferation and cell death is at least partially regulated by proto-oncogenes. A proto-oncogene can encode proteins that induce cellular proliferation (*e.g.*, *sis*, *erbB*, *src*, *ras* and *myc*), proteins that inhibit cellular proliferation 25 (*e.g.*, *Rb*, *p53*, *NF1* and *WT1*) or proteins that regulate programmed cell death (*e.g.*, *bcl-2*) (Ochi *et al.*, 1998; Johnson and Hamdy, 1998; Liebermann *et al.*, 1998). However, genetic rearrangements or mutations to these proto-oncogenes, results in the conversion of a proto-oncogene into a potent cancer causing oncogene. Often, a single point mutation is enough to transform a proto-oncogene into an oncogene. For example, a

mutation at codon 12 or 13 in the K-ras gene can convert the proto-oncogene into an oncogene.

Currently, there are few effective options for the treatment of many common cancer types. The course of treatment for a given individual depends on the diagnosis, the stage to which the disease has developed and factors such as age, sex and general health of the patient. The most conventional options of cancer treatment are surgery, radiation therapy and chemotherapy. Surgery plays a central role in the diagnosis and treatment of cancer. Typically, a surgical approach is required for biopsy and to remove cancerous growth. However, if the cancer has metastasized and is widespread, surgery is unlikely to result in a cure and an alternate approach must be taken. Radiation therapy, chemotherapy and immunotherapy are alternatives to surgical treatment of cancer (Mayer, 1998; Ohara, 1998; Ho *et al.*, 1998). Radiation therapy involves a precise aiming of high energy radiation to destroy cancer cells and much like surgery, is mainly effective in the treatment of non-metastasized, localized cancer cells. Side effects of radiation therapy include skin irritation, difficulty swallowing, dry mouth, nausea, diarrhea, hair loss and loss of energy (Curran, 1998; Brizel, 1998).

Chemotherapy, the treatment of cancer with anti-cancer drugs, is another mode of cancer therapy. The effectiveness of a given anti-cancer drug therapy is often limited by the difficulty of achieving drug delivery throughout solid tumors (el-Kareh and Secomb, 1997). Chemotherapeutic strategies are based on tumor tissue growth, wherein the anti-cancer drug is targeted to the rapidly dividing cancer cells. Most chemotherapy approaches include the combination of more than one anti-cancer drug, which has proven to increase the response rate of a wide variety of cancers (U.S. Patent 5,824,348; U.S. Patent 5,633,016 and U.S. Patent 5,798,339). A major side effect of chemotherapy drugs is that they also affect normal tissue cells, with the cells most likely to be affected being those that divide rapidly (*e.g.*, bone marrow, gastrointestinal tract, reproductive system and hair follicles). Other toxic side effects of chemotherapy drugs are sores in the mouth, difficulty swallowing, dry mouth, nausea, diarrhea, vomiting, fatigue, bleeding, hair loss

and infection. Other forms of chemotherapy can be used to treat non-cancerous hyperproliferative disorders. These include the use of conventional chemotherapeutics such as methotrexate and cyclophosphamide for hyperproliferative diseases such as rheumatoid arthritis and psoriasis. Chemotherapy for hyperproliferative diseases can also 5 include immunosuppressive agents such as steroids, azathioprine, cyclosporine and immunomodulatory agents such as fumaric acid derivatives.

The lipid drug delivery can also be used in combination with phetodynamic therapy or in combination with retinoids, biological therapies such as monoclonal and 10 cytokine antagonists (e.g., TNF-alpha receptor antagonist) or enzyme inhibitors such as difluromethyl or nithine. Inhibitors of matrix metalloproteinases could also be used in combination with the invention described (e.g., bromocriptin and butyred).

15 Immunotherapy, a rapidly evolving area in cancer research, is yet another option for the treatment of certain types of cancers. For example, the immune system identifies tumor cells as being foreign and thus they are targeted for destruction by the immune system. Unfortunately, the response typically is not sufficient to prevent most tumor growths. However, recently there has been a focus in the area of immunotherapy to develop methods that augment or supplement the natural defense mechanism of the 20 immune system. Examples of immunotherapies currently under investigation or in use are immune adjuvants (e.g., *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene and aromatic compounds) (U.S. Patent 5,801,005; U.S. Patent 5,739,169; Hui and Hashimoto, 1998; Christodoulides *et al.*, 1998), cytokine therapy (e.g., interferons α , β and γ ; IL-1, GM-CSF and TNF) (Bukowski *et al.*, 1998; Davidson 25 *et al.*, 1998; Hellstrand *et al.*, 1998) gene therapy (e.g., TNF, IL-1, IL-2, p53) (Qin *et al.*, 1998; Austin-Ward and Villaseca, 1998; U.S. Patent 5,830,880 and U.S. Patent 5,846,945) and monoclonal antibodies (e.g., anti-ganglioside GM2, anti-HER-2, anti-p185) (Pietras *et al.*, 1998; Hanibuchi *et al.*, 1998; U.S. Patent 5,824,311).

As mentioned above, proto-oncogenes play an important role in cancer biology. For example, Rb, p53, NF1 and WT1 tumor suppressors are essential for the maintenance of the non-tumorigenic phenotype of cells (reviewed by Soddu and Sacchi, 1998). Approximately 50% of all cancers have been found to be associated with mutations of the 5 p53 gene, which result in the loss of p53 tumor suppressor properties (Levine *et al.*, 1991; Vogelstein and Kinzler, 1992; Hartmann *et al.*, 1996a; Hartmann *et al.*, 1996b). The high incidence of mutations of the p53 gene in cancer has prompted many research groups to investigate p53 as a route of cancer treatment via gene transfer or replacement. The 10 proto-oncogenes *sis*, *erbB*, *src*, *ras* and *myc*, encoding proteins that induce cellular proliferation, and the proto-oncogenes of the Bcl-2 family that regulate programmed cell death also play important roles in the non-tumorigenic phenotype of cells.

Lipid delivery of nucleic acids for gene transfer or replacement therapy may be used alone, or in combination with the above described treatments to provide a means to 15 sensitize hyperproliferative and/or neoplastic cells to conventional therapies. Systemic delivery of nucleic acids will have the potential to target metastatic as well as solid tumor cancer and cancerous, pre-cancerous, and non-cancerous cells.

The optimization of lipid drug delivery by systemic administration would permit 20 the treatment of hyperproliferative cells throughout the organism. Therefore, there exists a need for a pharmaceutical lipid formulation that is capable of efficient nucleic acid transfer for the treatment of hyperproliferative diseases. Further, lipid drug delivery may increase the efficiency of gene transfer to target cells. Moreover, the need exists for a stable, pharmaceutically acceptable lipid delivery vehicle that is capable of delivering 25 pharmaceuticals, in particular nucleic acid based pharmaceuticals, for the treatment of hyperproliferative cells with altered expression of proto-oncogenes.

SUMMARY OF THE INVENTION

It is, therefore, an objective of the present invention to provide a pharmaceutically acceptable lipid formulation comprising DOTAP and at least one cholesterol or cholesterol derivative or cholesterol mixture in combination with a nucleic acid representing or encoding (a) an anti-proliferative protein or (b) an antisense molecule or a ribozyme that inhibits the expression of a growth-promoting protein for treating a subject with a hyperproliferative disease. It is contemplated that any of the formulations of the present invention may comprise an antisense molecule or ribozyme that inhibits the expression of a particular protein instead of a nucleic acid encoding an antisense molecule or ribozyme to a particular protein. It is further contemplated that any of the nucleic acids of the present invention may be under the control or operably linked to a promoter.

Additionally a dominant negative variant, a single chain antibody, or a decoy directed to an oncoprotein or growth-promoting protein can be delivered. The phrase "directed to" in the context of proteins and polypeptides "directed to" a targeted protein or polypeptide refer to the inhibition of activity, either directly or indirectly, of the targeted protein or polypeptide.

The formulation of a pharmaceutically acceptable lipid vehicle for the transfer of nucleic acids in the present invention comprises DOTAP and at least one cholesterol or cholesterol derivative or cholesterol mixture in combination with a nucleic acid representing or encoding an anti-proliferative protein or an antisense molecule or a ribozyme that inhibits the expression of growth-promoting protein. Additionally a dominant negative variant, a single chain antibody, or a decoy to an oncoprotein can also be delivered. Following administration of the formulation to a subject with a hyperproliferative disease, an expression construct comprising a therapeutic nucleic acid under the control of a promoter that is active in eukaryotic cells is transferred to cells via a lipid based vehicle. The therapeutic gene product may be expressed directly by the hyperproliferative cells, thereby stimulating a growth arrest or apoptic response.

Additionally or alternatively, it may be expressed by cells in the vicinity of the hyperproliferative cells to indirectly modulate their hyperproliferation. An example would be transfer of anti-proliferative or apoptotic genes to tumor vascular cells, thus leading to an anti-angiogenic process; another example involves a bystander effect in which a non-hyperproliferative cell expresses and secretes the therapeutic gene product such that nearby cells that do not express the product are similarly affected.

In certain preferred embodiments, the pharmaceutically acceptable lipid formulation includes DOTAP in a concentration ranging from 1 to 8 mM. In more preferred embodiments, the pharmaceutically acceptable lipid is a formulation comprising DOTAP in a concentration ranging from 2 to 7 mM. In still more preferred embodiments, the pharmaceutically acceptable lipid is a formulation comprising DOTAP in a concentration ranging from 3 to 6 mM. Most preferably, the pharmaceutically acceptable lipid is a formulation comprising DOTAP in a concentration of about 4 to 5 mM.

In certain preferred embodiments, the pharmaceutically acceptable lipid formulation includes cholesterol or cholesterol derivative or cholesterol mixture in a concentration ranging from 1 to 8 mM. In more preferred embodiments, the cholesterol or cholesterol derivative or cholesterol mixture is included in a concentration ranging from 2 to 7 mM. In still more preferred embodiments, the pharmaceutically acceptable lipid is a formulation comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration ranging from 3 to 6 mM. Most preferably the pharmaceutically acceptable lipid is a formulation comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration of about 4 to 5 mM.

Preferably, the DOTAP and cholesterol or cholesterol derivative or cholesterol mixture are included in a molar ratio of from about 3:1 to about 1:3, more preferably 2:1 to about 1:2, and most preferably about 1:1. It is also contemplated that the lipid formulation or liposome is extruded one or more times.

In preferred embodiments, the therapeutic nucleic acid is an oncogene, wherein the oncogene is a gene encoding a tumor suppressor, tumor associated protein, growth factor, growth-factor receptor, signal transducer, hormone, cell cycle regulator, nuclear factor, transcription factor or apoptotic factor. The therapeutic nucleic acid may encode a fusion protein that represents one or more of these species. The therapeutic nucleic acid may encode a polypeptide mimetic of one or more of these species. The therapeutic nucleic acid may be an "anti-oncogene," *i.e.*, a nucleic acid that produces a molecule that is able to abrogate or down-regulate the activity of proteins involved in increased cell proliferation, such as oncogenes or oncoproteins. Such molecules include antisense nucleic acids, ribozymes, and genes encoding single chain antibodies directed against oncoproteins, decoys for oncoproteins and dominant negative forms of oncoproteins. The therapeutic nucleic acids will also include therapeutic peptide nucleic acids and complexes of therapeutic nucleic acids with proteins such as transcription factors or polymerases. It is envisioned that therapeutic nucleic acids can be used in various combinations for the treatment of hyperproliferative diseases. Combinations of therapeutic nucleic acids can be delivered by a single lipid formulation or by administration of multiple lipid formulations. These combinations may include the genes for immunomodulatory molecules such as IL-2, F42K, GM-CSF, and/or Il-12. Additional genes include immunomodulatory antagonists (*e.g.*, TNF-alpha receptor antagonists).

In preferred embodiments, the anti-proliferative protein is a tumor suppressor protein, wherein the tumor suppressor is Rb, p53, p14, p15, p16, p19, INK4c, p21, p27, p73, a p16-p27 fusion, a p21-p27 fusion, p56^{RB}, E2F-1, NOEY2, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC, PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2, MDA-7, DBCCR-1, p57, Barx2, E1A, apotin, or WT-I. In particularly preferred embodiments, the tumor suppressor is p53. An "anti-proliferative protein" refers to a protein or polypeptide that is capable of reducing or inhibiting the growth, growth rate, or proliferation of a cell, including a

hyperproliferative cell or tumor cell. Anti-proliferative proteins include proteins that promote apoptosis.

In certain embodiments of the invention, an antisense molecule or ribozyme directed to a growth factor receptor encoding molecule. In further embodiments, an anti-proliferative protein is a single chain antibody, a decoy, or a dominant negative and is directed to a growth factor or growth factor receptor protein. In preferred embodiments, the growth factor receptor is FMS, ERBB/HER, ERBB-2/NEU/HER-2, ERBA, TGF- β receptor, PDGF receptor, MET, KIT or TRK.

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In another embodiment, the anti-proliferative protein is a single chain antibody, a decoy, or a dominant negative directed to a signal transducer protein or an antisense molecule or ribozyme directed to a signal transducer protein. In preferred embodiments, the signal transducer protein is SRC, ABL, RAS, AKT/PKB, RSK-1, RSK-2, RSK-3, 15 RSK-B, PRAD, LCK, or ATM.

In yet another embodiment of the invention, the anti-proliferative protein is a single chain antibody, a decoy, or a dominant negative directed to a transcription factor protein or an antisense molecule or ribozyme directed to a transcription factor protein. In preferred embodiments, the transcription factor protein is JUN, FOS, MYC, BRCA1, 20 BRCA2, ERBA, ETS, EVII, MYB, HMGI-C, HMGG/LIM, SKI, VHL, WT1, CEBP- α , NF κ B, I κ B, GL1, or REL.

Additional molecules that can be used in association with the invention are molecules that upregulate cell surface receptors for TRAIL, FAS ligand, and/ or other surface receptors for apoptosis inducing ligands or the ligands themselves, angiogenesis inhibitors, and anti-thrombic agents or genes.

In yet another embodiment, the anti-proliferative protein is a single chain antibody, a decoy, or a dominant negative directed to a growth factor protein or an

antisense molecule or ribozyme directed to a growth factor protein. In preferred embodiments, the growth factor protein is SIS, HST, INT-1/WT1, or INT-2.

In certain embodiments, the anti-proliferative protein is an apoptotic factor protein, wherein the apoptotic factor protein is Bax, Bak, Bim, Bik, Bcl-xL, Bcl-xS, Bid, Bad, Bcl-2, Harakiri, or an ICE protease.

In certain embodiments, the anti-proliferative protein is a single chain antibody, a decoy, or a dominant negative directed to a tumor associated protein or an antisense molecule or ribozyme directed to a tumor-associated protein, wherein the tumor associated protein is CEA, mucin, MAGE, or GAGE.

In certain preferred embodiments, the pharmaceutically acceptable lipid formulation further comprises a targeting moiety. More preferred embodiments include a targeting moiety that is a peptide, a ligand, or an antibody. In still more preferred embodiments, the peptide includes a RGD or GFE sequence. In certain preferred embodiments the peptide is from 3 to 30 amino acids in length, more preferred is from 3 to 20 amino acids in length, and most preferred is from 4 to 10 amino acids in length. More preferably, the peptide is a cyclic peptide.

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In certain preferred embodiments, the targeting moiety comprises a ligand which is a substrate for a cell surface protein. In further preferred embodiments, the ligand is a substrate for integrins, proteoglycans, glycoproteins, receptors, and transporters. In still further preferred embodiments, the targeting moiety comprises an antibody that binds to a cell surface protein. Most preferably, the antibody is an antibody to the Her-1 receptor.

The therapeutic nucleic acid may be linear, branched, or circular. It may be single-, double-, or triple-stranded or a mixture. In certain embodiments, the therapeutic nucleic acid contains a promoter. The promoter may be isolated from any natural source, 30 may be synthetic, or may be chimeric, i.e., pieces of it may come from multiple sources

including synthesis. In certain preferred embodiments, the promoter is CMV IE, VEGF, CEA, dectin-1, dectin-2, human CD11c, F4/80, SM22-alpha or MHC class II. More preferably the promoter is CMV IE. In another preferred embodiment the expression vector further comprises a polyadenylation signal. The therapeutic nucleic acid may also 5 contain a transcriptional enhancer. The enhancer may be isolated from any natural source, may be totally synthetic, or may be chimeric, *i.e.*, pieces of it may come from multiple sources including synthesis. In certain preferred embodiments the enhancer is derived from CMV. The therapeutic nucleic acid may also contain an intron. The intron 10 may be isolated from any natural source, maybe synthetic, or may be chimeric, *i.e.*, pieces of it may come from multiple sources including synthesis. In certain preferred embodiments, the intron is derived from CMV. The therapeutic nucleic acid may be non-replicating, conditionally-replicating, or replicating in the host cell. The therapeutic nucleic acid may include protein secretory signals or translocation sequences that permit its protein product to be passed from one cell to another cell or to the circulation.

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It is contemplated, of this present invention, a method of treating hyperproliferative disorders comprising administering an effective amount of the lipid formulation to a patient in need of anti-proliferative therapy. In certain preferred embodiments, the hyperproliferative disease of interest can be any disorder in which the 20 normal cell homeostasis is altered and an imbalance of cell proliferation and cell death leads to a dysregulated cellular proliferation. The aforementioned hyperproliferation can lead to neoplastic, pre-neoplastic and non-neoplastic disease states. It is further contemplated that a hyperproliferative neoplastic disease is cancer, wherein the cancer is derived from the dysregulated proliferation of a somatic, stem or germ cell from various 25 tissues. Examples of cancer include, but are not limited to lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, ovarian, leukemia, myeloma, esophageal, skin, thyroid, liver, brain, colorectal and bladder cancer.

In certain preferred embodiments, the hyperproliferative disease is non-cancerous. 30 In further preferred embodiments, non-cancerous hyperproliferative diseases include but

are not limited to rheumatoid arthritis, cardiac disease, adenoma, leiomyoma, lipoma, hemangioma, fibroma, inflammatory bowel disease, osteo-arthritis, and psoriasis. It is still further contemplated that the hyperproliferative disorder is vascular occlusion disease, more preferably vascular restenosis.

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Also contemplated in the present invention are various treatment methods of contacting the target cells with a pharmaceutically acceptable lipid formulation. Included in these treatment methods is intravenous, intradermal, intra-arterial, intra-peritoneal, 10 intraleisional, intracranial, intraarticular, intraprostatic, intrapleural, intratracheal, intramuscular, intranasal, intravesicular, intravitreal, intravaginal, rectal and intratumoral injection or administration; infusion, continuous infusion; localized perfusion; bathing target cells directly or via a catheter; topical administration; or transdermal absorption; and/or aerosolization, instillation. In certain preferred embodiments, the formulation is administered to the tumor bed prior to or after resection of the tumor.

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It is envisioned that treatments will be either single or multiple administrations via one or a combination of the aforementioned treatment methods. Treatments will be administered directly, locally, regionally, and/or systemically in order to contact the target cells.

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It is contemplated, of the present invention, that the formulation is administered to the patient before, during or after chemotherapy, surgery or radiotherapy. Preferably the lipid formulation is administered less than 72 hours prior to chemotherapy, surgery or radiotherapy. More preferably the lipid formulation is administered less than 24 hours 25 prior to chemotherapy, surgery or radiotherapy. In another preferred embodiment the lipid formulation is administered less than 72 hours after chemotherapy, surgery or radiotherapy. More preferably the lipid formulation is administered less than 24 hours after chemotherapy, surgery or radiotherapy. It further contemplated that the invention may be practiced as a therapy in and of itself.

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Disseminated hyperproliferative diseases are commonly treated with chemotherapeutic agents. Characteristics of chemotherapeutic agents limit the time and magnitude of patient exposure. It is contemplated, of the present invention, that the effectiveness of chemotherapy can be enhanced by combining chemotherapeutic and lipid formulation treatments. Combination chemotherapies include, for example, cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, mitomycin, etoposide, tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, taxotere, and methotrexate or any analog or derivative variant thereof.

As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least 15 a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Therapeutic effect of *p53* and *FHIT* complexed to DOTAP:Chol DNA:liposome treatment on subcutaneous human lung cancer xenografts. FIG. 1a. Subcutaneous H1299 tumor-bearing mice were divided into three groups (8 animals/group) and treated daily for a total of six doses (100 µg/dose), as follows: no treatment (♦), *p53* plasmid DNA (□) and DOTAP:Chol-*p53* DNA:liposome complex (■) (FIG. 1a); no treatment (♦), DOTAP:Chol-*p4d* DNA:liposome complex (□) and DOTAP:Chol-*p53* DNA:liposome complex (■) (FIG. 1b). Tumors were measured using calipers and statistical significance calculated using Student's *t* test. Each time point represents the average mean tumor volume for each group. Bars represent standard error.

10 FIG. 2b. *p53* gene expression and apoptotic cell death following DOTAP:Chol-*p53* DNA:liposome treatment. Subcutaneous H1299 tumors from animals receiving no treatment, *p53* plasmid, or the DOTAP:Chol-*p53* DNA:liposome complex were harvested at 48 hours after treatment. *p53* protein production was analyzed by immunohistochemistry and apoptotic cell death by TUNEL staining. The percentage of cells producing the *p53* protein (39%) (FIG. 1c) and undergoing apoptotic cell death (32%) (FIG. 1d) in tumors receiving the DOTAP:Chol-*p53* DNA:liposome complex was significant ($p = 0.001$) versus the percentage of cells in the no treatment and *p53* plasmid treatment groups. FIG. 1e-f. Therapeutic effect of the DOTAP:Chol-*FHIT* DNA:liposome complex on lung tumor xenografts. Subcutaneous H1299 and A549 tumor-bearing nude mice were divided into four groups (7 animals/group) and treated daily for a total of six doses (100 µg/dose), as follows: no treatment, *FHIT* plasmid DNA, the DOTAP:Chol-*CAT* DNA:liposome complex, and DOTAP:Chol-*FHIT* DNA:liposome complex. H1299 tumor treatments (FIG. 1e), and A549 tumor treatments (FIG. 1f). Tumor growth was significantly inhibited in H1299 ($p = 0.02$) and A549 ($p = 0.001$) tumor-bearing animals treated with the DOTAP:Chol-*FHIT* DNA:liposome complex. Bars denote standard error.

FIG. 2. Effects of intravenous treatment with extruded DOTAP:Chol-*p53* DNA:liposome complex or DOTAP:Chol-*FHIT* DNA:liposome complex on lung metastases. FIG. 2a. Inhibition of H1299 lung metastases following DOTAP:Chol-*p53*

DNA:liposome complex treatment. H1299 lung tumor-bearing SCID/Beige mice were either not treated or treated daily for a total of six doses (50 µg/dose) with *p53* plasmid DNA, the DOTAP:DOPE-*p53* DNA:liposome complex, the nonextruded DOTAP:Chol-*p53* DNA:liposome complex, the extruded DOTAP:Chol-*CAT* DNA:liposome complex, or the extruded DOTAP:Chol-*p53* DNA:liposome complex. Each group was comprised of eight animals. Metastatic tumor growth ($p = 0.001$) was significantly inhibited in mice treated with extruded DOTAP:Chol-*p53* DNA:liposome complex as compared with growth in the other groups (FIG. 2a). FIG. 2b. Inhibition of A549 lung metastases following DOTAP:Chol-*p53* DNA:liposome complex treatment. A599 lung tumor-bearing nu/nu mice were either not treated or treated daily for a total of six doses (50 µg/dose) with the extruded DOTAP:Chol-*CAT* DNA:liposome complex, or the extruded DOTAP:Chol-*p53* DNA:liposome complex. Each group was comprised of eight animals. Metastatic tumor growth ($p = 0.001$) was significantly inhibited in mice treated with extruded DOTAP:Chol-*p53* DNA:liposome complex as compared with growth in the two control groups. FIG. 2c. Inhibition of A549 lung metastases following DOTAP:Chol-*FHIT* DNA:liposome complex treatment. A549 lung tumor-bearing nu/nu mice were divided into four groups and treated as follows: no treatment, treatment with *FHIT* plasmid DNA, treatment with DOTAP:Chol-*CAT* DNA:liposome complex, and treatment with DOTAP:Chol-*FHIT* DNA:liposome complex daily for a total of six doses (50 µg/dose). Each group comprised of six animals. There was significant reduction in the number of lung tumor ($p = 0.007$) in mice treated with DOTAP:Chol-*FHIT* DNA:liposome complex. In all the experiments, lungs were harvested 2 weeks after the last treatment and metastases counted without knowledge of the treatment group. Bars denote standard error.

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FIG 3. Survival experiments in mice treated with DOTAP:Chol-*p53* DNA:liposome complex treatments. Female SCID/Beige mice were injected with 10^6 H1299 tumor cells (six animals/group) and BALB/c nu/nu mice were injected with 10^6 A549 tumor cells (seven animals/group) via the tail vein. Animals were divided into four groups and treated daily, as follows: no treatment, treatment with *p53* plasmid DNA,

treatment with the DOTAP:Chol-CAT DNA:liposome complex, or treatment with the DOTAP:Chol-p53 DNA:liposome complex. Animals in each group received a total of six doses (50 µg/dose) and were monitored daily to assess morbidity and mortality. Animal survival was estimated using the Kaplan-Meier and Wilcoxon signed rank tests.

5 Survival was significantly increased in H1299 lung tumor-bearing animals treated with the DOTAP:Chol-p53 DNA:liposome complex ($p = 0.001$) as compared with survival in control animals that received either no treatment or treatment with p53 plasmid DNA or the DOTAP:Chol-CAT DNA:liposome complex (FIG. 3a). H1299 injected animals showed tumor dissemination to various organs and tissues, including the cervical lymph

10 nodes, colon, mesentery, and liver. Survival was also significantly increased in A549 lung tumor-bearing mice treated with the DOTAP:Chol-p53 DNA:liposome complex ($p = 0.04$) as compared with survival in animals from control groups (FIG. 3b).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

15

The present invention contemplates the formulation of a pharmaceutically acceptable lipid, comprising DOTAP and cholesterol, a cholesterol derivative or a cholesterol mixture, and a nucleic acid encoding an anti-proliferative protein, antisense or ribozyme for delivery of the nucleic acid into hyperproliferative cells or into cells near the hyperproliferative cells. The treatment of such a hyperproliferative disease in one embodiment involves the intravenous administration of a lipid and a p53 expression construct complex to hyperproliferative cells, which subsequently express the p53 wild-type protein. The hyperproliferative cells then express p53, resulting in the stasis, destruction or lysis of hyperproliferative cells.

20

25

A. Hyperproliferative Disease

A variety of hyperproliferative diseases can be treated according to the methods of the present invention. Some of the hyperproliferative diseases contemplated for treatment in the present invention are psoriasis, rheumatoid arthritis (RA), vascular occlusion, 5 including vascular restenosis, inflammatory bowel disease (IBD), osteoarthritis (OA) and pre-neoplastic lesions in the mouth, prostate, breast, lung, etc. Also included are adenoma, leiomyoma, lipoma, hemangioma, and fibroma. The present invention has important ramifications particularly with respect to one hyperproliferative disease: cancer.

10

Cancer has become one of the leading causes of death in the Western world, second only behind heart disease. Current estimates project that one person in three in the U.S. will develop cancer, and that one person in five will die from cancer. Cancers can be viewed as altered cells that have lost the normal growth-regulating mechanisms.

15

There are currently three major categories of oncogenes, reflecting their different activities. One category of oncogenes encode proteins that induce cellular proliferation. A second category of oncogenes, called tumor-suppressor genes or anti-oncogenes, functions to inhibit excessive cellular proliferation. The third category of oncogenes 20 either blocks or induces apoptosis by encoding proteins that regulate programmed cell death. Members within each of these categories can be toxic when expressed in cells. This toxicity may be prohibitive during the production of viral based pharmaceuticals in mammalian systems. The inherent toxicity of these nucleic acids is not an issue in regards to the manufacturing processes of the present invention. It is envisioned, of the 25 present invention, to be used in the manufacturing of said toxic nucleic acids. This does not limit the present invention to the manufacture of the toxic nucleic acids as less toxic or non-toxic nucleic acids may be manufactured as well.

In some embodiments, the treatment of a wide variety of cancerous states or 30 tissue/organ types is within the scope of the invention, for example, melanoma, non-small

cell lung, small-cell lung, lung, hepatocarcinoma, retinoblastoma, astrocytoma, glioblastoma, leukemia, blood, brain, skin, eye, tongue, gum, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon or bladder. In still more preferred embodiments, 5 the hyperproliferative disease being treated according to the present invention is rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, leiomyomas, ademonas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, pre-neoplastic lesions, carcinoma *in situ*, oral hairy leukoplakia or psoriasis.

10 In one embodiment of the present invention, the treatment of hyperproliferative disease involves the intravenous administration of a therapeutic nucleic acid expression construct to treat hyperproliferative cells. It is contemplated that the hyperproliferative cells will express the nucleic acid, thus restoring a missing function, supplementing an existing function, reducing a proliferative function, or adding a new protein that results in 15 the growth arrest, destruction or lysis of hyperproliferative cells; such a nucleic acid would encode an anti-proliferative protein. An anti-proliferative polypeptide or protein is one that reduces or inhibits the growth, proliferation, or growth rate of a cell, or one that promotes or increases apoptosis of a cell. Alternatively, in other embodiments of the invention described below, an antisense molecule or ribozyme that inhibits the expression 20 of a growth promoting polypeptide may be used to treat a hyperproliferative disease. A growth-promoting protein or polypeptide increases or promotes the growth rate, proliferation, or growth of a cell. It is contemplated that a nucleic acid molecule encoding such an antisense or ribozyme may be employed as well. The three major categories of oncogenes are discussed below and listed in Table 1.

25

1. Inducers of Cellular Proliferation

The proteins that induce or promote cellular proliferation (growth) fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. For example, a form of PDGF, the sis 30 oncogene, is a secreted growth factor. Oncogenes rarely arise from genes encoding

growth factors, and at the present, sis is the only known naturally occurring oncogenic growth factor. In one embodiment of the present invention, it is contemplated that anti-sense mRNA, or a ribozyme, directed to a particular inducer of cellular proliferation (growth-promoting protein) is used to prevent expression of the inducer of cellular proliferation.

The proteins fms, erbA, erbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation affecting the transmembrane domain of the neu receptor protein results in the neu oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic erbA receptor is believed to compete with the endogenous thyroid hormone receptor, causing uncontrolled growth.

The largest class of oncogenes is the signal transducing proteins (*e.g.*, src, abl and ras). The protein src, is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino acid 12 in the sequence, reducing ras GTPase activity.

20

The proteins jun, fos and myc are proteins that directly exert their effects on nuclear functions as transcription factors. Table 1 lists a variety of the oncogenes described in this section and many of those not described.

25

2. Inhibitors of Cellular Proliferation

The tumor suppressor oncogenes function to inhibit excessive cellular proliferation (*i.e.*, anti-proliferative protein). The inactivation of these genes destroys or reduces their inhibitory activity, resulting in unregulated proliferation. Thus, wild-type version of these tumor suppressors inhibit proliferation, and consequently are anti-proliferative. The tumor suppressors p53, p16 and C-CAM are described below.

High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is 5 already documented to be the most frequently-mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes 10 with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations that are minute by comparison with transformed cells or tumor tissue.

Wild-type p53 is recognized as an important growth regulator in many cell types. 15 Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, 20 dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G₁ phase. The 25 activity of this enzyme may be to phosphorylate Rb at late G₁. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the p16^{INK4}. p16^{INK4} has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the p16^{INK4} protein is a CDK4 inhibitor (Serrano, 1993), 30 deletion of this gene may increase the activity of CDK4, resulting in

hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

5 p16^{INK4} belongs to a newly described class of CDK-inhibitory proteins that also includes p16^B, p21^{WAF1}, p57^{KIP2} and p27^{KIP1}. The p16^{INK4} gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16^{INK4} gene are frequent in human tumor cell lines. This evidence suggests that the p16^{INK4} gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the p16^{INK4} gene alterations
10 is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type p16^{INK4} function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto,
15 1994; Arap, 1995).

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kDa, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.
25

Cell adhesion molecules, or CAMs are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAMs may be involved in the tumorigenesis of several neoplasms; for example, decreased expression of
30 E-cadherin, which is predominantly expressed in epithelial cells, is associated with the

progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matsura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells *in vivo*. C-
5 CAM now has been shown to suppress tumor growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include p14, p15, p19, GAX, p56^{RB}, INK4c, RB, APC, FHIT, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1, mda-7, DBCCR-1, FCC and MCC (see
10 Table 1).

3. Regulators of Programmed Cell Death

Apoptosis, or programmed cell death, is an essential occurring process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing
15 carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986;
20 Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins that can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death
25 triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, Bcl_{xL}, Bcl_w, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri, Bcl_{Xs}). Proteins that

promote cell death are anti-proliferative proteins; conversely, cells that suppress apoptosis are growth-promoting proteins.

TABLE 1
ONCOGENES

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
Growth Factors			
<i>HST/KS</i>	Transfection		FGF family member
<i>INT-2</i>	MMTV promoter Insertion		FGF family member
<i>INT1/WNT1</i>	MMTV promoter Insertion		Factor-like
<i>SIS</i>	Simian sarcoma virus		PDGF B
Receptor Tyrosine Kinases			
<i>ERBB/HER</i>	Avian erythroblastosis virus; ALV promoter insertion; amplified human tumors	Amplified, deleted squamous cell cancer; glioblastoma	EGF/TGF- α / amphiregulin/ hetacellulin receptor
<i>ERBB-2/NEU/HER-2</i>	Transfected from rat Glioblatoms	Amplified breast, ovarian, gastric cancers	Regulated by NDF/ heregulin and EGF- related factors
<i>FMS</i>	SM feline sarcoma virus		CSF-1 receptor
<i>KIT</i>	HZ feline sarcoma virus		MGF/Steel receptor hematopoieis
<i>TRK</i>	Transfection from human colon cancer		NGF (nerve growth factor) receptor
<i>MET</i>	Transfection from human osteosarcoma		Scatter factor/HGF receptor
<i>RET</i>	Translocations and point mutations	Sporadic thyroid cancer; familial medullary thyroid cancer; multiple endocrine neoplasias 2A and 2B	Orphan receptor Tyr kinase
<i>ROS</i>	URII avian sarcoma Virus		Orphan receptor Tyr kinase
<i>PDGF receptor</i>	Translocation	Chronic	TEL(ETS-like

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
		myelomonocytic leukemia	transcription factor)/ PDGF receptor gene fusion
<i>TGF-β</i> receptor		Colon carcinoma mismatch mutation target	

NONRECEPTOR TYROSINE KINASES

<i>ABL</i>	Abelson MuLV	Chronic myelogenous leukemia translocation with BCR	Interact with RB, RNA polymerase, CRK, CBL
<i>FPS/FES</i>	Avian Fujinami SV; GA FeSV		
<i>LCK</i>	MuLV (murine leukemia virus) promoter insertion		Src family; T cell signaling; interacts CD4/CD8 T cells
<i>SRC</i>	Avian Rous sarcoma Virus		Membrane-associated Tyr kinase with signaling function; activated by receptor kinases
<i>YES</i>	Avian Y73 virus		Src family; signaling

SER/THR PROTEIN KINASES

<i>AKT</i>	AKT8 murine retrovirus	Regulated by PI(3)K?; regulate 70-kd S6 k?
<i>MOS</i>	Maloney murine SV	GVBD; cystostatic factor; MAP kinase kinase
<i>PIM-1</i>	Promoter insertion Mouse	
<i>RAF/MIL</i>	3611 murine SV; MH2 avian SV	Signaling in RAS pathway

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
MISCELLANEOUS CELL SURFACE			
<i>APC</i>	Tumor suppressor	Colon cancer	Interacts with catenins
<i>DCC</i>	Tumor suppressor	Colon cancer	CAM domains
E-cadherin	Candidate tumor Suppressor	Breast cancer	Extracellular homotypic binding; intracellular interacts with catenins
<i>PTC/NBCCS</i>	Tumor suppressor and <i>Drosophila</i> homology	Nevvoid basal cell cancer syndrome (Gorline syndrome)	12 transmembrane domain; signals through Gli homologue Cl to antagonize hedgehog pathway
<i>TAN-1</i> Notch homologue	Translocation	T-ALI.	Signaling?
MISCELLANEOUS SIGNALING			
<i>BCL-2</i>	Translocation	B-cell lymphoma	Apoptosis
<i>CBL</i>	Mu Cas NS-1 V		Tyrosine- phosphorylated RING finger interact Abl
<i>CRK</i>	CT1010 ASV		Adapted SH2/SH3 interact Abl
<i>DPC4</i>	Tumor suppressor	Pancreatic cancer	TGF- β -related signaling pathway
<i>MAS</i>	Transfection and Tumorigenicity		Possible angiotensin receptor
<i>NCK</i>			Adaptor SH2/SH3
GUANINE NUCLEOTIDE EXCHANGERS AND BINDING PROTEINS³			
<i>BCR</i>		Translocated with ABL in CML	Exchanger; protein kinase
<i>DBL</i>	Transfection		Exchanger
<i>GSP</i>			
<i>NF-1</i>	Hereditary tumor Suppressor	Tumor suppressor neurofibromatosis	RAS GAP

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
<i>OST</i>	Transfection		Exchanger
Harvey-Kirsten, N-RAS	HaRat SV; Ki RaSV; Balb-MoMuSV; Transfection	Point mutations in many human tumors	Signal cascade
<i>VAV</i>	Transfection		S112/S113; exchanger

NUCLEAR PROTEINS AND TRANSCRIPTION FACTORS

<i>BRCA1</i>	Heritable suppressor	Mammary cancer/ovarian cancer	Localization unsettled
<i>BRCA2</i>	Heritable suppressor	Mammary cancer	Function unknown
<i>ERBA</i>	Avian erythroblastosis Virus		thyroid hormone receptor (transcription)
<i>ETS</i>	Avian E26 virus		DNA binding
<i>EVII</i>	MuLV promotor insertion	AML	Transcription factor
<i>FOS</i>	FBI/FBR murine osteosarcoma viruses		1 transcription factor with c-JUN
<i>GLI</i>	Amplified glioma	Glioma	Zinc finger; cubitus interruptus homologue is in hedgehog signaling pathway; inhibitory link PTC and hedgehog
<i>HMGG/LIM</i>	Translocation t(3:12) t(12:15)	Lipoma	Gene fusions high mobility group HMGI-C (XT-hook) and transcription factor LIM or acidic domain
<i>JUN</i>	ASV-17		Transcription factor AP-1 with FOS
<i>MLL/VHRX + ELI/MEN</i>	Translocation/fusion ELL with MLL trithorax-like gene	Acute myeloid leukemia	Gene fusion of DNA- binding and methyl transferase MLL with ELI RNA pol II

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
			elongation factor
<i>MYB</i>	Avian myeloblastosis Virus		DNA binding
<i>MYC</i>	Avian MC29; translocation B-cell lymphomas; promoter insertion avian leukosis virus	Burkitt's lymphoma	DNA binding with MAX partner; cyclin regulation; interact RB?; regulate apoptosis?
<i>N-MYC</i>	Amplified	Neuroblastoma	
<i>L-MYC</i>		Lung cancer	
<i>REL</i>	Avian Reticuloendotheliosis virus		NF- κ B family transcription factor
<i>SKI</i>	Avian SKV770 Retrovirus		Transcription factor
<i>VHL</i>	Heritable suppressor	Von Hippel-Landau syndrome	Negative regulator or elongin; transcriptional elongation complex
<i>WT-1</i>		Wilm's tumor	Transcription factor
CELL CYCLE/DNA DAMAGE RESPONSE			
<i>ATM</i>	Heredity disorder	Ataxia-telangiectasia	Protein/lipid kinase homology; DNA damage response upstream in P53 pathway
<i>BCL-2</i>	Translocation	Follicular lymphoma	Apoptosis
<i>FACC</i>	Point mutation	Fanconi's anemia group C (predisposition leukemia)	
<i>FHIT</i>	Fragile site 3p14.2	Lung carcinoma	Histidine triad-related diadenosine 5',3''''- $P^1.p^4$ tetraphosphate asymmetric hydrolase

<i>Gene</i>	<i>Source</i>	<i>Human Disease</i>	<i>Function</i>
<i>hMLH/MutL</i>		HNPPCC	Mismatch repair; MutL homologue
<i>hMSH2/MutS</i>		HNPPCC	Mismatch repair; MutS homologue
<i>hPMS1</i>		HNPPCC	Mismatch repair; MutL homologue
<i>hPMS2</i>		HNPPCC	Mismatch repair; MutL homologue
<i>INK4/MTS1</i>	Adjacent INK-4B at 9p21; CDK complexes	Candidate MTS1 suppressor and MLM melanoma gene	p16 CDK inhibitor
<i>INK4B/MTS2</i>		Candidate suppressor	p15 CDK inhibitor
<i>MDM-2</i>	Amplified	Sarcoma	Negative regulator p53
<i>p53</i>	Association with SV40 T antigen	Mutated >50% human tumors, including hereditary Li-Fraumeni syndrome	Transcription factor; checkpoint control; apoptosis
<i>PRAD1/BCL1</i>	Translocation with parathyroid hormone or IgG	Parathyroid adenoma; B-CLL	Cyclin D
<i>RB</i>	Hereditary Retinoblastoma; association with many DNA virus tumor Antigens	Retinoblastoma; osteosarcoma; breast cancer; other sporadic cancers	Interact cyclin/cdk; regulate E2F transcription factor
<i>XPA</i>		xeroderma pigmentosum; skin cancer predisposition	Excision repair; photo-product recognition; zinc finger

4. Non-Cancer Hyperproliferative Diseases

In one embodiment of the present invention, it is contemplated that non-cancer hyperproliferative diseases may be treated by administering a therapeutic nucleic acid expression construct capable of eliciting an anti-hyperproliferative response. Some of the 5 hyperproliferative diseases contemplated for treatment in the present invention are psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), osteoarthritis (OA), adenoma, fibroma, hemangioma, lipoma, and pre-neoplastic lesions in the lung.

B. Liposome Mediated Transformation

10 Liposomes are vesicular structures characterized by a lipid bilayer and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when lipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of structures that entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Lipophilic molecules or molecules with lipophilic regions may 15 also dissolve in or associate with the lipid bilayer.

1. Liposome Preparation

The present invention relates to liposomes of a specific composition which form a 20 stable structure and are efficient carriers of biologically active agents. The liposomes are capable of carrying biologically active nucleic acids, such that the nucleic acids are completely sequestered. The liposome may contain one or more nucleic acids and is administered to a mammalian host to efficiently deliver its contents to a target cell. The 25 liposomes in the present invention comprise DOTAP and cholesterol or a cholesterol derivative. In certain embodiments, the ratio of DOTAP to cholesterol, cholesterol derivative or cholesterol mixture is about 10:1 to about 1:10, about 9:1 to about 1:9, about 8:1 to about 1:8, about 7:1 to about 1:7, about 6:1 to about 1:6, about 5:1 to about 1:5, about 4:1 to about 1:4, about 3:1 to 1:3, more preferably 2:1 to 1:2, and most 30 preferably 1:1. In further preferred embodiments, the DOTAP and/or cholesterol concentrations are about 1mM, 2 mM, 3mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM,

10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 25 mM, or 30 mM. The DOTAP and/or Cholesterol concentration can be between about 1 mM to about 20mM, 1 mM to about 18 mM, 1 mM to about 16 mM, about 1mM to about 14mM, about 1 mM to about 12 mM, about 1 mM to about 10 mM, 1 to 8 mM, 5 more preferably 2 to 7 mM, still more preferably 3 to 6 mM and most preferably 4 to 5 mM. Cholesterol derivatives may be readily substituted for the cholesterol or mixed with the cholesterol in the present invention. Many cholesterol derivatives are known to the skilled artisan. Examples include but are not limited to cholesterol acetate and cholesterol oleate. A cholesterol mixture refers to a composition that contains at least one 10 cholesterol or cholesterol derivative.

The formulation may also be extruded using a membrane or filter, and this may be performed multiple times. Such techniques are well-known to those of skill in the art, for example in Martin (1990). Extrusion may be performed to homogenize the formulation 15 or limit its size.

Formulations of the present invention are extremely stable and can complex nucleic acids over a wide range of nucleic acid: liposome ratios. This "range" allows optimization of the complexes for delivery *in vivo*. The stability of DOTAP:Cholesterol 20 liposomes at high concentrations of liposome and DNA allows further for increased concentrations of DNA for delivery and expression.

A contemplated method for preparing liposomes in certain embodiments is heating, sonicating, and sequential extrusion of the lipids through filters of decreasing 25 pore size, thereby resulting in the formation of small, stable liposome structures. This preparation produces liposomal complexes or liposomes only of appropriate and uniform size, which are structurally stable and produce maximal activity.

For example, it is contemplated in certain embodiments of the present invention 30 that DOTAP:Cholesterol liposomes are prepared by the methods of Templeton *et al.*

(1997; incorporated herein by reference). Thus, in one embodiment, DOTAP (cationic lipid) is mixed with cholesterol (neutral lipid) at equimolar concentrations. This mixture of powdered lipids is then dissolved with chloroform, the solution dried to a thin film and the film hydrated in water containing 5% dextrose (w/v) to give a final concentration of
5 20 mM DOTAP and 20 mM cholesterol. The hydrated lipid film is rotated in a 50°C water bath for 45 minutes, then at 35°C for an additional 10 minutes and left standing at room temperature overnight. The following day the mixture is sonicated for 5 minutes at 50°C. The sonicated mixture is transferred to a tube and heated for 10 minutes at 50°C. This mixture is sequentially extruded through syringe filters of decreasing pore size (1
10 μm, 0.45 μm, 0.2 μm, 0.1 μm).

It also is contemplated that other liposome formulations and methods of preparation may be combined to impart desired DOTAP:Cholesterol liposome characteristics. Alternate methods of preparing lipid-based formulations for nucleic acid delivery are described by Saravolac *et al.* (W0 99/18933). Detailed are methods in which lipids compositions are formulated specifically to encapsulate nucleic acids. In another liposome formulation, an amphipathic vehicle called a solvent dilution microcarrier (SDMC) enables integration of particular molecules into the bi-layer of the lipid vehicle (U.S. Patent 5,879,703). The SDMCs can be used to deliver lipopolysaccharides, polypeptides, nucleic acids and the like. Of course, any other methods of liposome preparation can be used by the skilled artisan to obtain a desired liposome formulation in the present invention.
15
20

2. Liposome-Mediated Nucleic Acid Delivery

In certain embodiments, anti-hyperproliferative genes are delivered *in vivo* via liposomes to treat hyperproliferative diseases. The liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured
25 chick embryo, HeLa and hepatoma cells.
30

In one example of the present invention, the liposome complex (DNA:lipid complex or DNA lipoplex) is prepared by diluting a given nucleic acid and lipids in 5% dextrose in water to obtain an appropriate concentration of nucleic acid and lipids. Equal 5 volumes of nucleic acid and lipids, at a concentration to obtain about 25 µg, 50 µg, 75 µg, 100 µg, 110 µg, 120 µg, 125 µg, 130 µg, 140 µg, 150 µg, 160 µg, 170 µg, 180 µg, 190 µg, 200 µg, 210 µg, 220 µg, 225 µg, 230 µg, 240 µg, 250 µg, 260 µg, 270 µg, 275 µg, 280 µg, 290 µg; 300 µg, 310 µg, 320 µg, 325 µg, 330 µg, 340 µg, 350 µg, 360 µg, 370 µg, 375 µg, 400 µg, 425 µg, 450 µg, 500µg, 550 µg, 600 µg, 650 µg, 700 µg, 750 µg, 800 µg, 850 µg, 900 µg, 950 µg, or 1000 µg of nucleic acid per 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, 20 mM, 22 mM, 24 mM, 26 mM, 28 mM, 30 mM, 32 mM, 34 mM, 36 mM, 38 mM, or 40 mM lipids per 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 15 5000, 5500, 6000, 7000, 8000, 9000, or 10,000 µl, as well as 15, 20, 25, or 50 ml is mixed by adding the nucleic acid rapidly to the surface of the lipid solution followed by two rapid up and down expulsions to effect rapid mixing.

Various nucleic acids may be added to these liposomes in a wide range of 20 concentrations. Nucleic acids are preferably added to the liposomes at a concentration of 20, 25, 50, 75, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 275, 300, 350, 375, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 µg per 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 7000, 8000, 9000, or 10,000 µl, as well 25 as 15, 20, 25, 50 ml final volume. These concentrations vary widely depending upon the ratio of DOTAP to cholesterol, cholesterol derivative or cholesterol mixture in the particular liposome preparation. The actual dosage amount of liposome-nucleic acid administered to a patient can be determined by physical and physiological factors such as body weight, severity of condition, idiopathy of the patient and on the route of

administration. With these considerations in mind, the dosage of liposome-nucleic acid complex for a particular subject and/or course of treatment can readily be determined.

As described in other sections, the liposomes of the present invention containing nucleic acid can be administered intravenously, intradermally, intra-arterially, intra-peritoneally, intralesionally, intracranially, intra-articularly, intraprostaticaly, intrapleurally, intratracheally, intranasally, intravitrealy, intravaginaly, rectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, intravesicularily, mucosally, intrapericardially, orally and/or using aerosol, injection, infusion, continuous infusion, localized perfusion bathing target cells directly or via a catheter and/or lavage.

3. Targeting Moieties for Liposome Delivery

The present invention provides also a targeting means, such that the liposomes can be delivered to specific cell types. Because the nucleic acid is sequestered in the liposome's cavity, targeted delivery is achieved by the addition of ligands without compromising the ability of these liposomes to bind and deliver large amounts of the nucleic acid. It is contemplated that this will enable delivery to specific organs and tissues. The targeting specificity of the ligand-based delivery systems are based on the distribution of the ligand receptors on different cell types. The targeting ligand may either be non-covalently or covalently associated with the lipid complex.

Targeting ligands are any ligand specific for a characteristic component of the targeted region. Preferred targeting ligands include proteins such as polyclonal or monoclonal antibodies, antibody fragments, or chimeric antibodies, enzymes, or hormones, or sugars such as mono-, oligo- and poly-saccharides. In certain embodiments of the invention, contemplated targeting ligands interact with integrins, proteoglycans, glycoproteins, receptors or transporters. Suitable ligands include any that are specific for cells of the target organ, or for structures of the target organ exposed to the circulation as a result of local pathology, such as tumors.

In certain embodiments of the present invention, in order to enhance the transduction of resistant cells, to increase transduction of target cells, or to limit transduction of undesired cells, antibody or cyclic peptide targeting moieties (ligands) are associated with the lipid complex. The antibody targeting moiety in particular examples 5 is a monoclonal anti-EGF receptor antibody. EGF stimulates cell growth and proliferation through interaction with an EGF receptor. EGF receptors are distributed on the cell surface of various organs and are present in burns, wounds, dermis and tumors. In particular embodiments, the peptide targeting moiety is a cyclic peptide containing 10 within its sequence a RGD integrin binding motif. Ligands such as the RGD peptide that bind to integrins on the cell surface can mediate internalization, thus increasing the efficiency of delivery of the targeted complex. The targeting peptide may include an RGDFV sequence, wherein the peptide includes the RGD sequence in which the peptide is from 3 to 30 amino acids in length. In other embodiments the RGD integrin binding motif is from 3 to 20 amino acids in length or 4 to 10 amino acids in length. In particular 15 embodiments of the present invention, the RGD integrin binding motif is a peptide of 5 amino acids in length. Although cyclic peptides which contain the RGD integrin binding motif within its sequence are preferred, linear peptides may also be utilized in the present invention. Ligands may be identified by panning phage display libraries against cells and/or tissues of interest.

20

Contemplated also in the present invention is a targeting moiety comprising an EGF receptor binding peptide ligand (e.g., EGF or an EGF fragment).

Liposomes have been described further that specifically target cells of the 25 mammalian central nervous system (U.S. Patent 5,786,214, specifically incorporated herein by reference in its entirety). The liposomes are composed essentially of N-glutarylphosphatidylethanolamine, cholesterol and oleic acid, wherein a monoclonal antibody specific for neuroglia is conjugated to the liposomes.

The present invention further contemplates that the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1.

Antibodies described by Nicholson *et al.* (U.S. Patent 5,902,584), which bind the G-CSF extracellular domain can be used in the present invention for targeting liposomes. Alternatively, monoclonal antibody fragments may be used to target delivery to specific organs in the animal including brain, heart, lungs or liver. An exemplary method for targeting viral particles to cells that lack a single cell-specific marker is described (U.S. Patent 5,849,718). For example, antibody A may have specificity for tumor, but also for normal heart and lung tissue, while antibody B has specificity for tumor but also normal liver cells. Clearly, the use of antibody A or antibody B alone to deliver an anti-proliferative nucleic acid to the tumor would possibly result in unwanted damage to heart and lung or liver cells. However, antibody A and antibody B can be used together for improved cell targeting. Thus, antibody A is coupled to a gene encoding an anti-proliferative nucleic acid and is delivered, via a receptor mediated uptake system, to tumor as well as heart and lung tissue. However, the gene is not transcribed in these cells as they lack a necessary transcription factor. Antibody B is coupled to a universally active gene encoding the transcription factor necessary for the transcription of the anti-proliferative nucleic acid and is delivered to tumor and liver cells. Therefore, in heart and lung cells only the inactive anti-proliferative nucleic acid is delivered, where it is not transcribed, leading to no adverse effects. In liver cells, the gene encoding the transcription factor is delivered and transcribed, but has no effect because no an anti-proliferative nucleic acid gene is present. In tumor cells, however, both genes are delivered and the transcription factor can activate transcription of the anti-proliferative nucleic acid, leading to tumor-specific toxic effects.

Many other ligands may be employed for this targeting step of liposome preparation, depending upon the site targeted for liposome delivery. In certain embodiments, it is contemplated that liposomes are targeted to specific cell types by receptor-mediated endocytosis. For example, lactosyl ceramide, and peptides that target the LDL receptor related proteins, such as apolipoprotein E3 ("Apo E") have been useful in targeting liposomes to the liver (Spanjer and Scherphof, 1983; WO 98/0748). The asialoglycoprotein, asialofetuin, which contains terminal galactosyl residues, also has been demonstrated to target liposomes to the liver (Spanjer and Scherphof, 1983; Hara *et al.*, 1996). The sugars mannosyl, fucosyl or N-acetyl glucosamine, when coupled to the backbone of a polypeptide, bind the high affinity manose receptor (U.S. Patent 5,432,260, specifically incorporated herein by reference in its entirety). Thus, these glycoproteins can be conjugated to liposomes of the present invention and are contemplated as useful for targeting specific cells (e.g., macrophages).

15

Folate and the folate receptor have also been described as useful for cellular targeting (U.S. Patent 5,871,727). In this example, the vitamin folate is coupled to the complex. The folate receptor has high affinity for its ligand and is overexpressed on the surface of several malignant cell lines, including lung, breast and brain tumors. Anti-folate such as methotrexate may also be used as targeting ligands. Transferrin mediated delivery systems target a wide range of replicating cells that express the transferrin receptor (Gilliland *et al.*, 1980).

The addition of targeting ligands for gene delivery for the treatment of hyperproliferative diseases permits the delivery of genes whose gene products are more toxic than do non-targeted systems. Examples of the more toxic genes that can be delivered includes pro-apoptotic genes such as Bax and Bak plus genes derived from viruses and other pathogens such as the adenoviral E4orf4 and the *E.coli* purine nucleoside phosphorylase, a so-called "suicide gene" which converts the prodrug 6-methylpurine deoxyriboside to toxic purine 6-methylpurine. Other examples of suicide

genes used with prodrug therapy are the *E. coli* cytosine deaminase gene and the HSV thymidine kinase gene.

It is also possible to utilize untargeted or targeted lipid complexes to generate
5 recombinant or modified viruses *in vivo*. For example, two or more plasmids could be used to introduce retroviral sequences plus a therapeutic gene into a hyperproliferative cell. Retroviral proteins provided in *trans* from one of the plasmids would permit packaging of the second, therapeutic gene-carrying plasmid. Transduced cells, therefore,
10 would become a site for production of non-replicative retroviruses carrying the therapeutic gene. These retroviruses would then be capable of infecting nearby cells. The promoter for the therapeutic gene may or may not be inducible or tissue specific.

Similarly, the transferred nucleic acid may represent the DNA for a replication competent or conditionally replicating viral genome, such as an adenoviral genome that
15 lacks all or part of the adenoviral E1a or E2b region or that has one or more tissue-specific or inducible promoters driving transcription from the E1a and/or E1b regions. This replicating or conditional replicating nucleic acid may or may not contain an additional therapeutic gene such as a tumor suppressor gene or anti-oncogene.

20 **5. Liposome-Ligand Conjugation**

The liposomes of the invention can be targeted to specific regions of the body by attachment of specific targeting ligands to provide rapid accumulation and concentration of liposomes and, correspondingly, of nucleic acid molecules, in a designated tissue. The ligands contemplated for use in the present invention can be conjugated to the liposomes
25 by a variety of methods.

Bifunctional cross-linking reagents have been extensively used for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural
30 studies. Homobifunctional reagents that carry two identical functional groups proved to

be highly efficient in inducing cross-linking between identical and different macromolecules or subunits of a macromolecule, and linking of polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of the differential reactivities of the two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino, sulfhydryl, guanidino, indole, carboxyl specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group.

Exemplary methods for cross-linking ligands to liposomes are described in U.S. Patent 5,603,872 and U.S. Patent 5,401,511, each specifically incorporated herein by reference in its entirety). Various ligands can be covalently bound to liposomal surfaces through the cross-linking of amine residues. Liposomes, in particular, multilamellar vesicles (MLV) or unilamellar vesicles such as microemulsified liposomes (MEL) and large unilamellar liposomes (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. The inclusion of PE in the liposome provides an active functional residue, a primary amine, on the liposomal surface for cross-linking purposes. Ligands such as epidermal growth factor (EGF) have been successfully linked with PE-liposomes. Ligands are bound covalently to discrete sites on the liposome surfaces. The number and surface density of these sites will be dictated by the liposome formulation and the liposome type. The liposomal surfaces may also have sites for non-covalent association. To form covalent conjugates of ligands and liposomes, cross-linking reagents have been studied for effectiveness and biocompatibility. Cross-linking reagents include glutaraldehyde (GAD), bifunctional oxirane (OXR), ethylene glycol diglycidyl ether (EGDE), and a water soluble carbodiimide, preferably 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).

Through the complex chemistry of cross-linking, linkage of the amine residues of the recognizing substance and liposomes is established.

In another example, heterobifunctional cross-linking reagents and methods of
5 using the cross-linking reagents are described (U.S. Patent 5,889,155, specifically
incorporated herein by reference in its entirety). The cross-linking reagents combine a
nucleophilic hydrazide residue with an electrophilic maleimide residue, allowing
coupling in one example, of aldehydes to free thiols. The cross-linking reagent can be
modified to cross-link various functional groups and is thus useful for cross-linking
10 polypeptides and sugars. Table 2 details certain hetero-bifunctional cross-linkers
considered useful in the present invention.

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PCT/US00/14350

TABLE 2
HETERO-BIFUNCTIONAL CROSS-LINKERS

linker	Reactive Toward	Advantages and Applications	Spacer Arm Length/after cross-linking
SMPT	Primary amines Sulphydryls	· Greater stability	11.2 Å
SPDP	Primary amines Sulphydryls	· Thiolation · Cleavable cross-linking	6.8 Å
LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm	15.6 Å
Sulfo-LC-SPDP	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	15.6 Å
SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Enzyme-antibody conjugation · Hapten-carrier protein conjugation	11.6 Å
Sulfo-SMCC	Primary amines Sulphydryls	· Stable maleimide reactive group · Water-soluble · Enzyme-antibody conjugation	11.6 Å
MBS	Primary amines Sulphydryls	· Enzyme-antibody conjugation · Hapten-carrier protein conjugation	9.9 Å
Sulfo-MBS	Primary amines Sulphydryls	· Water-soluble	9.9 Å
SIAB	Primary amines Sulphydryls	· Enzyme-antibody conjugation	10.6 Å
Sulfo-SIAB	Primary amines Sulphydryls	· Water-soluble	10.6 Å
SMPB	Primary amines Sulphydryls	· Extended spacer arm · Enzyme-antibody conjugation	14.5 Å
Sulfo-SMPB	Primary amines Sulphydryls	· Extended spacer arm · Water-soluble	14.5 Å
EDC/Sulfo-NHS	Primary amines Carboxyl groups	· Hapten-Carrier conjugation	0
ABH	Carbohydrates Nonselective	· Reacts with sugar groups	11.9 Å

In instances where a particular polypeptide does not contain a residue amenable for a given cross-linking reagent in its native sequence, conservative genetic or synthetic amino acid changes in the primary sequence can be utilized.

5 The targeting ligand can be either anchored in the hydrophobic portion of the complex or attached to reactive terminal groups of the hydrophilic portion of the complex. The targeting ligand can be attached to the liposome via a linkage to a reactive group, *e.g.*, on the distal end of the hydrophilic polymer. Preferred reactive groups include amino groups, carboxylic groups, hydrazide groups, and thiol groups. The
10 coupling of the targeting ligand to the hydrophilic polymer can be performed by standard methods of organic chemistry that are known to those skilled in the art. The total concentration of the targeting ligand can be from 0.01 to 10% mol.

6. **Vectors and Regulatory Signals**

15 Vectors of the present invention are designed, primarily, to transform hyperproliferative cells with a therapeutic gene under the control of regulated eukaryotic promoters (*i.e.*, inducible, repressible, tissue-specific) or to transform nearby cells, such as tumor vasculature. Also, the vectors usually will contain a selectable marker if, for no other reason, to facilitate their production *in vitro*. However, selectable markers may play
20 an important role in producing recombinant cells and thus a discussion of promoters is useful here. Table 3 and Table 4 below, list inducible promoter elements and enhancer elements, respectively.

7. **Eukaryotic and Viral Promoters and Enhancers**

25 Preferred for use in the present invention is the cytomegalovirus (CMV) promoter. This promoter is commercially available from Invitrogen in the vectors pcDNAIII and pVAX1, which are preferred for use in the present invention. Also contemplated as useful in the present invention are the dectin-1 and dectin-2 promoters. Below are a list of additional viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the present invention.
30

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding oligosaccharide processing enzymes, protein folding accessory proteins, selectable marker proteins or a heterologous protein of interest.

5

Another signal that may prove useful is a polyadenylation signal (e.g., hGH, BGH, SV40).

The use of internal ribosome binding sites (IRES) elements are used to create
10 multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow,
15 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

20

Table 3 - Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter <i>et al.</i> , 1982; Haslinger and Karin, 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987; Karin ®, 1987; Angel <i>et al.</i> , 1987b; McNeill <i>et al.</i> , 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors and Varmus, 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Fonta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1986
β-Interferon	poly(rI)X poly(rc)	Tavernier <i>et al.</i> , 1983
Adenovirus 5 <u>E2</u>	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987a
Stromelysin	Phorbol Ester (TPA)	Angle <i>et al.</i> , 1987b
SV40	Phorbol Ester (TFA)	Angel <i>et al.</i> , 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez <i>et al.</i> , 1988
α-2-Macroglobulin	IL-6	Kunz <i>et al.</i> , 1989
Vimentin	Serum	Rittling <i>et al.</i> , 1989
MHC Class I Gene H-2kb	Interferon	Blanar <i>et al.</i> , 1989
HSP70	Ela, SV40 Large T Antigen	Taylor <i>et al.</i> , 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel <i>et al.</i> , 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee <i>et al.</i> , 1989

Table 4 - Other Promoter/Enhancer Elements

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Hanerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1988; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> , 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria <i>et al.</i> , 1987; Winoto and Baltimore, 1989; Redondo <i>et al.</i> , 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β -Interferon	Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene <i>et al.</i> , 1989
Interleukin-2 Receptor	Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990
MHC Class II 5	Koch <i>et al.</i> , 1989
MHC Class II HLA-DR α	Sherman <i>et al.</i> , 1989
β -Actin	Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> , 1989
Muscle Creatine Kinase	Jaynes <i>et al.</i> , 1988; Horlick and Benfield, 1989; Johnson <i>et al.</i> , 1989a
Prealbumin (Transthyretin)	Costa <i>et al.</i> , 1988
Elastase I	Omitz <i>et al.</i> , 1987
Metallothionein	Karin <i>et al.</i> , 1987; Culotta and Hamer, 1989
Collagenase	Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987
Albumin Gene	Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990
α -Fetoprotein	Godbout <i>et al.</i> , 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β -Globin	Trudel and Constantini, 1987
e-fos	Cohen <i>et al.</i> , 1987
c-HA-ras	Triesman, 1986; Deschamps <i>et al.</i> , 1985

Promoter/Enhancer	References
Insulin	Edlund <i>et al.</i> , 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch <i>et al.</i> , 1990
α_1 -Antitrypsin	Latimer <i>et al.</i> , 1990
H2B (TH2B) Histone	Hwang <i>et al.</i> , 1990
Mouse or Type I Collagen	Ripe <i>et al.</i> , 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang <i>et al.</i> , 1989
Rat Growth Hormone	Larsen <i>et al.</i> , 1986
Human Serum Amyloid A (SAA)	Edbrooke <i>et al.</i> , 1989
Troponin I (TN I)	Yutzey <i>et al.</i> , 1989
Platelet-Derived Growth Factor	Pech <i>et al.</i> , 1989
Duchenne Muscular Dystrophy	Klamut <i>et al.</i> , 1990
SV40	Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987 Schaffner <i>et al.</i> , 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; deVilliers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a,b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander and Haseltine, 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky and Botchan, 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987, Stephens and Hentschel, 1987; Glue <i>et al.</i> , 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988

Promoter/Enhancer	References
Human Immunodeficiency Virus	Muesing <i>et al.</i> , 1987; Hauber and Cullan, 1988; Jakobovits <i>et al.</i> , 1988; Feng and Holland, 1988; Takebe <i>et al.</i> , 1988; Rowen <i>et al.</i> , 1988; Berkhouit <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp and Marciniak, 1989; Braddock <i>et al.</i> , 1989
Cytomegalovirus	Weber <i>et al.</i> , 1984; Boshart <i>et al.</i> , 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989

The promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each 5 element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the 10 thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 15 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl 20 transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that 5 promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

10 Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of 15 many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a 20 promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.

Promoters and enhancers have the same general function of activating 25 transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

In any event, it will be understood that promoters are DNA elements which when positioned functionally upstream of a gene leads to the expression of that gene. Most transgene constructs of the present invention are functionally positioned downstream of a promoter element.

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The nucleic acids of this invention may also contain one or more introns. The inclusion of an intron has been found in many cases to lead to increased levels of transgene expression. Introns may also be included that contain binding sites for transcription factors. The nucleic acid may include a leader sequence to enhance 10 translation such as the tripartite leader sequence of adenovirus.

The nucleic acids of this invention may be linear, branched, or circular. A preferred embodiment of the present invention utilizes plasmids that carry a eukaryotic expression cassette as well as prokaryotic sequences that allow replication in prokaryotic 15 production systems. These plasmids may be non-replicating in a mammalian host, conditionally replicating, or replicating. An example of replicating plasmids would be those that contain an origin of replication and EBNA1 gene from the Epstein Barr virus. An example of a conditionally replicating plasmid would be one in which the EBNA-1 gene was under the control of a cell-specific, tumor-specific, or inducible promoter.

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8. Antisense Molecules and Ribozymes

a. Antisense

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides 25 are those capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine,

6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. An antisense polynucleotide, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs or molecules, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. The antisense molecule, or the DNA encoding the antisense molecule is said to be "directed against" a nucleic acid or gene whose transcription or translation is targeted. That nucleic acid or gene may encode a particular type of polypeptide, such as a growth-promoting protein, which means the antisense molecule is intended to prevent the expression of a protein that promotes growth, for example a growth factor.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs may include regions complementary to 20 intron/exon splice junctions. Thus, antisense constructs with complementarity to regions within 50-200 bases of an intron-exon splice junction may be used. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much 25 exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences 30 that are substantially complementary over their entire length and have very few base

mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated.

5 For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

10

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

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B. RIBOZYMES

The use of specific ribozymes is claimed in the present application. The following information is provided in order to complement the earlier section and to assist those of skill in the art in this endeavor. Thus, ribozymes may also be directed against a particular nucleic acid, as were the antisense molecules discussed above.

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlack *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub,

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This specificity has been attributed to the requirement that the substrate bind *via*

specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cell lines to which they were applied; the altered genes included the oncogenes H-ras, c-fos and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. In light of the information included herein and the knowledge of one of ordinary skill in the art, the preparation and use of additional ribozymes that are specifically targeted to a given gene will now be straightforward.

Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples that would be expected to function equivalently for the down regulation of IGFBP-2 include sequences from the Group I self splicing introns including tobacco ringspot virus (Prody *et al.*, 1986), avocado sunblotch viroid (Palukaitis *et al.*, 1979 and Symons, 1981), and Lucerne transient streak virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozymes based on a predicted folded secondary structure.

25

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan *et al.*, 1992; Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz *et al.*, 1992; Chowrira *et al.*, 1993) and hepatitis delta virus based ribozymes (Perrotta and Been, 1992). The general design and optimization of ribozyme directed

RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988, Symons, 1992, Chowrira, *et al.*, 1994, and Thompson, *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a
5 given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to
a site by complimentary base pair interactions. Two stretches of homology are required
for this targeting. These stretches of homologous sequences flank the catalytic ribozyme
structure defined above. Each stretch of homologous sequence can vary in length from 7
to 15 nucleotides. The only requirement for defining the homologous sequences is that,
10 on the target RNA, they are separated by a specific sequence which is the cleavage site.
For hammerhead ribozymes, the cleavage site is a dinucleotide sequence on the target
RNA, uracil (U) followed by either an adenine, cytosine or uracil (A,C or U; Perriman,
et al., 1992; Thompson, *et al.*, 1995). The frequency of this dinucleotide occurring in any
given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of
15 1000 bases, 187 dinucleotide cleavage sites are statistically possible. The message for
IGFBP-2 targeted here are greater than 1400 bases long, with greater than 260 possible
cleavage sites.

Designing and testing ribozymes for efficient cleavage of a target RNA is a
20 process well known to those skilled in the art. Examples of scientific methods for
designing and testing ribozymes are described by Chowrira *et al.*, (1994) and Lieber and
Strauss (1995), each incorporated by reference. The identification of operative and
preferred sequences for use in targeted ribozymes is simply a matter of preparing and
testing a given sequence, and is a routinely practiced "screening" method known to those
25 of skill in the art.

9. Single-Chain Antibodies, Decoys and Dominant Negatives

Another embodiment of the present invention contemplates the use a nucleic acid
encoding a single-chain antibody, a decoy or a dominant negative directed at particular
30 type of polypeptide in cells, in particular hyperproliferative cells, to block that

polypeptide's activity. Single-chain antibodies, decoys, and dominant negatives can be synthesized by a cell, targeted to particular cellular compartments, and used to interfere in a highly specific manner with cell growth and metabolism (Richardson and Marasco, 1995).

5

Recently, single-chain antibodies were utilized for the phenotypic knockout of growth-factor receptors, the functional inactivation of p21ras, and the inhibition of HIV-1 replication. Intracellular antibodies offer a simple and effective alternative to other forms of gene inactivation, as well as demonstrate a clear potential as reagents for cancer therapy and for the control of infectious diseases. Single-chain antigen-binding proteins also represent potentially unique molecules for targeted delivery of drugs, toxins, or radionuclides to a tumor site, and show increased accessibility to tumor cells *in vivo* (Yokoda *et al.*, 1992). Single-chain antibodies that bind a growth-promoting protein can be introduced into a cell to functionally inactivate the protein.

15

Methods for the production of single-chain antibodies are well known to those of skill in the art. The skilled artisan is referred to U.S. Patent 5,359,046, (incorporated herein by reference) for such methods. A single-chain antibody is created by fusing together the variable domains of the heavy and light chains using a short peptide linker, thereby reconstituting an antigen binding site on a single molecule.

Single-chain antibody variable fragments (scFvs) in which the C-terminus of one variable domain is tethered to the N-terminus of the other *via* a 15 to 25 amino acid peptide or linker, have been developed without significantly disrupting antigen binding or specificity of the binding (Bedzyk *et al.*, 1990; Chaudhary *et al.*, 1990). These Fvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody.

Decoys and dominant negatives are polypeptides that prevent a particular polypeptide from effecting its activity. Typically, they bind the targeted polypeptide or the polypeptide against which they are directed. A decoy is all or part of a polypeptide

that lures the targeted polypeptide away, to prevent the targeted polypeptide from interacting with other polypeptides with which it normally interacts. Binding of the decoy and the targeted polypeptide may sequester the targeted polypeptide such that it can no longer reach a particular part of the cell.

5

A dominant negative is all or part of a polypeptide that exerts a dominant effect on the targeted polypeptide and prevents it from functioning properly. For example, if the targeted polypeptide functions as a dimer (hetero- or homo-), a dominant negative could comprise the dimerization domain of another polypeptide that interacts with the targeted polypeptide but otherwise lack the remaining functional domains of the other polypeptide, such as the transducing domain. The dominant negative (dimerization domain) would bind to the targeted polypeptide, but the complex would be unable to form an active complex since other functional domains are absent. Many growth factor receptors and signal transducers, such as KIT, PDGF, FGF, and FMS, require dimerization to be able to respond to growth factors, and many transcription factors, such as JUN, FOS, and MYC, are functional only as dimers to bind DNA. Binding may also prevent the targeted polypeptide from selectively interacting with another polypeptide with which it usually interacts.

20 It is also contemplated by the present invention that therapies involving single-chain antibodies, decoys, and dominant negatives can be combined with other anti-cancer therapies, such as chemotherapy, radiotherapy, surgery, immunotherapy, or hormone therapy. The discussion of combined therapy with traditional anti-cancer therapies employed herein is incorporated into this section by reference.

25

C. Pharmaceutical Formulations and Nucleic Acid Delivery

In a preferred embodiment of the present invention, a formulation of a pharmaceutically acceptable lipid for the delivery of therapeutic nucleic acids for the treatment of hyperproliferative diseases is contemplated. Hyperproliferative diseases that

are most likely to be treated in the present invention are those that result from mutations in an oncogene and/or the reduced expression of a wild type protein in the hyperproliferative cells. Examples of hyperproliferative diseases contemplated for treatment are lung cancer, head and neck cancer, breast cancer, pancreatic cancer, prostate
5 cancer, renal cancer, bone cancer, testicular cancer, cervical cancer, gastrointestinal cancer, lymphomas, pre-neoplastic lesions in the lung, head and neck, cervix, intestine, pancreas and other organs; as well as colorectal cancer, breast cancer, bladder cancer, and any other hyperproliferative diseases that may be treated by administering a nucleic acid encoding a protein with anti-proliferative properties. Examples of other
10 hyperproliferative conditions include but are not limited to adenomas, benign prostatic hypertrophy, intraepithelial neoplasia, endometriosis, and colonic polyps.

In certain embodiments, the present invention also concerns formulations of one or more nucleic acid compositions for administration to a mammal. For the treatment of
15 hyperproliferative disease in humans, it is contemplated that a pharmaceutical lipid formulation, comprising DOTAP and cholesterol, a cholesterol derivative or a cholesterol mixture, and a nucleic acid under the control of a promoter operable in eukaryotic cells (e.g., CMV IE, dectin-1, dectin-2) will be used. It will also be understood that, if desired, the lipid:nucleic acid compositions disclosed herein may be administered in combination
20 with other agents as well, e.g., various pharmaceutically-active agents. As long as the composition comprises at least one nucleic acid, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect to the organism upon administration.

25 The formulation of pharmaceutically-acceptable excipients and carrier solutions are well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including, e.g., intradermal, parenteral, intravenous, intramuscular, intranasal, intrapericardial, mucosal, topical, aerosol and oral administration and
30 formulation.

D. Injectable Compositions and Delivery

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a hyperproliferative cell with the therapeutic expression construct. The routes of administration will vary, naturally, with the location and nature of the lesion, and include, e.g., intradermal, transdermal, parenteral, intravenous, intramuscular, intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion, lavage, direct injection, and oral administration and formulation. The formulations of the invention may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more times. They may also be administered 1, 2, 3, 4, 5, 6, 7, 8 or more times a day, or they maybe administered every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, or 5 weeks, or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months.

15

Intratumoral injection, or injection into the tumor vasculature is specifically contemplated for discrete, solid, accessible tumors. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The liposome:nucleic acid may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

25

In the case of surgical intervention, the present invention may be used preoperatively, to render an inoperable tumor subject to resection. Alternatively, the present invention may be used at the time of surgery, and/or thereafter, to treat residual or metastatic disease. For example, a resected tumor bed may be injected or perfused with a DOTAP:Cholesterol formulation comprising a nucleic acid construct encoding an anti-proliferative protein. The perfusion may be continued post-resection, for example, by

leaving a catheter implanted at the site of the surgery. Periodic post-surgical treatment also is envisioned.

Continuous administration also may be applied where appropriate, for example,
5 where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion
10 will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

Treatment regimens may vary as well, and often depend on tumor type, tumor location, disease progression, and health and age of the patient. Obviously, certain types
15 of tumor will require more aggressive treatment, while at the same time, certain patients cannot tolerate more taxing protocols. The clinician will be best suited to make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations.

20 In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic liposomal formulations may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional treatments subsequent to resection will serve to eliminate microscopic residual
25 disease at the tumor site.

A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose application over a two-week period. The two-week regimen may be repeated one, two,

three, four, five, six or more times. During a course of treatment, the need to complete the planned dosings may be re-evaluated.

The preferred method of the lipid : nucleic acid expression construct delivery to hyperproliferative cells in the present invention is via intravenous injection. However, the pharmaceutical compositions disclosed herein may alternatively be administered parenterally, intradermally, intramuscularly, or even intraperitoneally as described in U.S. Patent 5,543,158; U.S. Patent 5,641,515 and U.S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Injection of nucleic acid constructs may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions or liposomal suspension, particularly multi-vial formulations, of the active compounds as free base or pharmacologically acceptable salts may be prepared in water, in buffered solutions, and suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or

dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin. For multi-viral formulations the carrier compounds, the liposome, prepared in sterile suspensions that are suitable for deliver to humans.

For single viral formulations, suspensions of the active compounds in the liposome carrier with and without additional components may be used as described in the instant application.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration,

preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds and carriers in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, prepared by aseptic procedure and/or followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of associated protein(s)) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except

insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5 The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable
10 for solution in, or suspension in, liquid prior to injection can also be prepared.

E. Additional Modes of Delivery

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of therapeutic nucleic acid delivery.
15 Sonophoresis (*i.e.*, ultrasound) has been used and described in U.S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Similarly, electroporation has been used to deliver genes *in vivo* (Suzuki *et al.*, 1998). Other drug delivery alternatives contemplated are intraosseous injection (U.S. Patent 5,779,708),
20 microchip devices (U.S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U.S. Patent 5,770,219 and U.S. Patent 5,783,208), rectal delivery (U.S. Patent 5,811,128) and feedback controlled delivery (U.S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

F. Pharmaceuticals and Methods of Treating Cancer

In a particular aspect, the present invention provides methods for the treatment of various hyperproliferative diseases. Treatment methods will involve treating an individual with an effective amount of a lipid formulation, as described above, containing a nucleic acid of interest. An effective amount is described, generally, as that amount sufficient to detectably and repeatedly ameliorate, reduce, minimize or limit the extent of the disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

In order to increase the effectiveness of a liposome:nucleic acid complex it may be desirable to combine these compositions with other agents effective in the treatment of hyperproliferative disease, such as anti-cancer agents. An "anti-cancer" agent is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer. Anti-cancer agents include biological agents (biotherapy), chemotherapy agents, and radiotherapy agents. More generally, these other compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the expression construct and the agent(s) or multiple factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the expression construct and the other includes the second agent(s).

Tumor cell resistance to chemotherapy and radiotherapy agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For

example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, *et al.*, 1992). In the context of the present invention, it is contemplated that anti-proliferative gene therapy could be used similarly in conjunction 5 with chemotherapeutic, radiotherapeutic, or immunotherapeutic intervention, in addition to other pro-apoptotic or cell cycle regulating agents.

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor or tissue size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the 10 methods and compositions of the present invention, one would generally contact a hyperproliferative cell with the therapeutic expression construct. This may be combined with compositions comprising other agents effective in the treatment of hyperproliferative cells. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the 15 expression construct and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes a nucleic acid and the other includes the second agent.

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Alternatively, the lipid complex therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and nucleic acid are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the 25 agent and nucleic acid would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one may contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks 30 (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, gene therapy is "A" and the radio- or chemotherapeutic other therapeutic agent is "B":

5 A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

10

Administration of the therapeutic nucleic acid constructs of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of the vector. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described lipid formulation therapy.

20 Aqueous compositions of the present invention comprise an effective amount of the compound, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

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The treatments may include various "unit doses." Unit dose is defined as containing a predetermined quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of import is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of nucleic acid mass (μg) of the nucleic acid in the lipid complex. Unit doses range from 1, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 300, 400, 500, 600, 700, 800, 900, 1000 μg and higher.

Preferably, patients will have adequate bone marrow function (defined as a peripheral absolute granulocyte count of $> 2,000 / \text{mm}^3$ and a platelet count of $100,000 / \text{mm}^3$), adequate liver function (bilirubin $< 1.5 \text{ mg / dl}$) and adequate renal function (creatinine $< 1.5 \text{ mg / dl}$).

One of the preferred embodiments of the present invention involves the use of lipid nucleic acid complex to deliver therapeutic genes to hyperproliferative cells for the treatment of cancer. Cancer cells include cancers of the lung, brain, prostate, kidney, liver, ovary, breast, skin, stomach, esophagus, head and neck, testicles, colon, rectum, cervix, lymphatic system and blood. Of particular interest are non-small cell lung carcinomas including squamous cell carcinomas, adenocarcinomas and large cell undifferentiated carcinomas.

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According to the present invention, one may treat the cancer by directly injecting a tumor with the lipid complex. Local or regional administration, with respect to the tumor, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease.

Delivery *via* syringe, infusion or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion 5 will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

For tumors of > 4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of < 4 cm, a volume of about 1-3 ml will be used 10 (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The lipid complex may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

In certain embodiments, the tumor being treated may not, at least initially, be 15 resectable. Treatments with therapeutic lipid complex formulations may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional lipid complex treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

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A typical course of treatment, for a primary tumor or a post-excision tumor bed, will involve multiple doses. Typical primary tumor treatment involves a 6 dose application over a two-week period. The two-week regimen may be repeated one, two, three, four, five, six or more times. During a course of treatment, the need to complete 25 the planned dosings may be re-evaluated. For systemic administration the liposome complexes may be administered from 0.5 to several hours by infusion with a pharmaceutically acceptable diluent such as 5% dextrose in water, Ringer's, 0.5% NaCl. The systemic administration may occur once per week for multiple weeks over the course of months.

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Preferably, patients will have adequate bone marrow function (defined as a peripheral absolute granulocyte count of > 2,000 / mm³ and a platelet count of 100,000 / mm³), adequate liver function (bilirubin < 1.5 mg / dl) and adequate renal function (creatinine < 1.5 mg / dl).

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1. Gene Therapy

In yet another embodiment, the secondary treatment is a secondary gene therapy in which a second therapeutic polynucleotide is administered before, after, or at the same time a first therapeutic polynucleotide. Alternatively, a single vector encoding both genes 10 may be used. A variety of proteins are encompassed within the invention, some of which have been described earlier in the instant application.

2. Chemotherapy

Cancer therapies also include a variety of combination therapies with both 15 chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan. A wide variety of chemotherapeutic agents may be used in combination with the use of nucleic acid molecules in a pharmaceutically acceptable lipid formulation in the present 20 invention. The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by 25 affecting nucleic acid synthesis. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, antitumor antibiotics, corticosteroid hormones, mitotic inhibitors, and nitrosoureas, and any analog or derivative variant thereof. It is contemplated that the nucleic acid formulations described herein can be 30 used in combination with one or more of these agents according to the present invention.

a. Alkylating agents

Alkylating agents are drugs that directly interact with genomic DNA to prevent the cancer cell from proliferating. This category of chemotherapeutic drugs represents 5 agents that affect all phases of the cell cycle, that is, they are not phase-specific. Alkylating agents can be implemented to treat chronic leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and particular cancers of the breast, lung, and ovary. They include: busulfan, chlorambucil, cisplatin, cyclophosphamide (cytoxan), dacarbazine, ifosfamide, mechlorethamine (mustargen), and melphalan. 10 Troglitazaone can be used to treat cancer in combination with any one or more of these alkylating agents, some of which are discussed below.

i. Busulfan

Busulfan (also known as myleran) is a bifunctional alkylating agent. Busulfan is 15 known chemically as 1,4-butanediol dimethanesulfonate.

Busulfan is not a structural analog of the nitrogen mustards. Busulfan is available in tablet form for oral administration. Each scored tablet contains 2 mg busulfan and the inactive ingredients magnesium stearate and sodium chloride.

20 Busulfan is indicated for the palliative treatment of chronic myelogenous (myeloid, myelocytic, granulocytic) leukemia. Although not curative, busulfan reduces the total granulocyte mass, relieves symptoms of the disease, and improves the clinical state of the patient. Approximately 90% of adults with previously untreated chronic 25 myelogenous leukemia will obtain hematologic remission with regression or stabilization of organomegaly following the use of busulfan. It has been shown to be superior to splenic irradiation with respect to survival times and maintenance of hemoglobin levels, and to be equivalent to irradiation at controlling splenomegaly.

ii. Chlorambucil

Chlorambucil (also known as leukeran) is a bifunctional alkylating agent of the nitrogen mustard type that has been found active against selected human neoplastic diseases. Chlorambucil is known chemically as 4-[bis(2-chlorethyl)amino] 5 benzenebutanoic acid.

Chlorambucil is available in tablet form for oral administration. It is rapidly and completely absorbed from the gastrointestinal tract. After single oral doses of 0.6-1.2 mg/kg, peak plasma chlorambucil levels are reached within one hour and the terminal 10 half-life of the parent drug is estimated at 1.5 hours. 0.1 to 0.2mg/kg/day or 3 to 6mg/m²/day or alternatively 0.4mg/kg may be used for antineoplastic treatment. Treatment regimes are well known to those of skill in the art and can be found in the "Physicians Desk Reference" and in "Remington's Pharmaceutical Sciences" referenced herein.

15

Chlorambucil is indicated in the treatment of chronic lymphatic (lymphocytic) leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma and Hodgkin's disease. It is not curative in any of these disorders but may produce clinically useful palliation.

20

iii. Cisplatin

Cisplatin has been widely used to treat cancers such as metastatic testicular or ovarian carcinoma, advanced bladder cancer, head or neck cancer, cervical cancer, lung cancer or other tumors. Cisplatin can be used alone or in combination with other agents, 25 with efficacious doses used in clinical applications of 15-20 mg/m² for 5 days every three weeks for a total of three courses. Exemplary doses may be 0.50 mg/m², 1.0mg/m², 1.50 mg/m², 1.75 mg/m², 2.0 mg/m², 3.0 mg/m², 4.0 mg/m², 5.0 mg/m², 10mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

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Cisplatin is not absorbed orally and must therefore be delivered *via* injection intravenously, subcutaneously, intratumorally or intraperitoneally.

iv. Cyclophosphamide

5 Cyclophosphamide is 2*H*-1,3,2-Oxazaphosphorin-2-amine, *N,N*-bis(2-chloroethyl)tetrahydro-, 2-oxide, monohydrate; termed Cytoxan available from Mead Johnson; and Neosar available from Adria. Cyclophosphamide is prepared by condensing 3-amino-1-propanol with *N,N*-bis(2-chlorethyl) phosphoramidic dichloride [(ClCH₂CH₂)₂N--POCl₂] in dioxane solution under the catalytic influence of 10 triethylamine. The condensation is double, involving both the hydroxyl and the amino groups, thus effecting the cyclization.

15 Unlike other β -chloroethylamino alkylators, it does not cyclize readily to the active ethyleneimonium form until activated by hepatic enzymes. Thus, the substance is stable in the gastrointestinal tract, tolerated well and effective by the oral and parental routes and does not cause local vesication, necrosis, phlebitis or even pain.

20 Suitable doses for adults include, orally, 1 to 5 mg/kg/day (usually in combination), depending upon gastrointestinal tolerance; or 1 to 2 mg/kg/day; 25 intravenously, initially 40 to 50 mg/kg in divided doses over a period of 2 to 5 days or 10 to 15 mg/kg every 7 to 10 days or 3 to 5 mg/kg twice a week or 1.5 to 3 mg/kg/day. A dose 250mg/kg/day may be administered as an antineoplastic. Because of gastrointestinal adverse effects, the intravenous route is preferred for loading. During maintenance, a leukocyte count of 3000 to 4000/mm³ usually is desired. The drug also sometimes is administered intramuscularly, by infiltration or into body cavities. It is available in dosage forms for injection of 100, 200 and 500 mg, and tablets of 25 and 50 mg the skilled artisan is referred to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61, incorporate herein as a reference, for details on doses for administration.

v. Melphalan

Melphalan, also known as alkeran, L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcolysin, is a phenylalanine derivative of nitrogen mustard. Melphalan is a bifunctional alkylating agent which is active against selective human neoplastic diseases. It is known chemically as 4-[bis(2-chloroethyl)amino]-L-phenylalanine.

Melphalan is the active L-isomer of the compound and was first synthesized in 1953 by Bergel and Stock; the D-isomer, known as medphalan, is less active against certain animal tumors, and the dose needed to produce effects on chromosomes is larger than that required with the L-isomer. The racemic (DL-) form is known as merphalan or sarcolysin. Melphalan is insoluble in water and has a pKa, of ~2.1. Melphalan is available in tablet form for oral administration and has been used to treat multiple myeloma.

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Available evidence suggests that about one third to one half of the patients with multiple myeloma show a favorable response to oral administration of the drug.

Melphalan has been used in the treatment of epithelial ovarian carcinoma. One commonly employed regimen for the treatment of ovarian carcinoma has been to administer melphalan at a dose of 0.2 mg/kg daily for five days as a single course. Courses are repeated every four to five weeks depending upon hematologic tolerance (Smith and Rutledge, 1975; Young *et al.*, 1978). Alternatively the dose of melphalan used could be as low as 0.05mg/kg/day or as high as 3mg/kg/day or any dose in between these doses or above these doses. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject

b. Antimetabolites

Antimetabolites disrupt DNA and RNA synthesis. Unlike alkylating agents, they specifically influence the cell cycle during S phase. They have been used to combat chronic leukemias in addition to tumors of breast, ovary and the gastrointestinal tract.

5 Antimetabolites include 5-fluorouracil (5-FU), cytarabine (Ara-C), fludarabine, gemcitabine, and methotrexate.

i. 5-Fluorouracil

10 5-Fluorouracil (5-FU) has the chemical name of 5-fluoro-2,4(1H,3H)-pyrimidinedione. Its mechanism of action is thought to be by blocking the methylation reaction of deoxyuridylic acid to thymidylic acid. Thus, 5-FU interferes with the synthesis of deoxyribonucleic acid (DNA) and to a lesser extent inhibits the formation of ribonucleic acid (RNA). Since DNA and RNA are essential for cell division and proliferation, it is thought that the effect of 5-FU is to create a thymidine deficiency

15 leading to cell death. Thus, the effect of 5-FU is found in cells that rapidly divide, a characteristic of metastatic cancers.

c. Antitumor Antibiotics

20 Antitumor antibiotics have both antimicrobial and cytotoxic activity. These drugs also interfere with DNA by chemically inhibiting enzymes and mitosis or altering cellular membranes. These agents are not phase specific so they work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. Examples of antitumor antibiotics include bleomycin, dactinomycin, daunorubicin, doxorubicin (Adriamycin), and idarubicin, some of which are discussed in more detail below. Widely used in

25 clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin, to 35-100 mg/m² for etoposide intravenously or orally.

i. Doxorubicin

Doxorubicin hydrochloride, 5,12-Naphthacenedione, (8s-cis)-10-[(3-amino-2,3,6-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-hydrochloride (hydroxydaunorubicin hydrochloride, 5 Adriamycin) is used in a wide antineoplastic spectrum. It binds to DNA and inhibits nucleic acid synthesis, inhibits mitosis and promotes chromosomal aberrations.

Administered alone, it is the drug of first choice for the treatment of thyroid adenoma and primary hepatocellular carcinoma. It is a component of 31 first-choice 10 combinations for the treatment of ovarian, endometrial and breast tumors, bronchogenic oat-cell carcinoma, non-small cell lung carcinoma, gastric adenocarcinoma, retinoblastoma, neuroblastoma, mycosis fungoides, pancreatic carcinoma, prostatic carcinoma, bladder carcinoma, myeloma, diffuse histiocytic lymphoma, Wilms' tumor, Hodgkin's disease, adrenal tumors, osteogenic sarcoma soft tissue sarcoma, Ewing's 15 sarcoma, rhabdomyosarcoma and acute lymphocytic leukemia. It is an alternative drug for the treatment of islet cell, cervical, testicular and adrenocortical cancers. It is also an immunosuppressant.

Doxorubicin is absorbed poorly and must be administered intravenously. The 20 pharmacokinetics are multicompartmental. Distribution phases have half-lives of 12 minutes and 3.3 hr. The elimination half-life is about 30 hr. Forty to 50% is secreted into the bile. Most of the remainder is metabolized in the liver, partly to an active metabolite (doxorubicinol), but a few percent is excreted into the urine. In the presence of liver impairment, the dose should be reduced.

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Appropriate doses are, intravenous, adult, 60 to 75 mg/m² at 21-day intervals or 25 to 30 mg/m² on each of 2 or 3 successive days repeated at 3- or 4-wk intervals or 20 mg/m² once a week. The lowest dose should be used in elderly patients, when there is prior bone-marrow depression caused by prior chemotherapy or neoplastic marrow 30 invasion, or when the drug is combined with other myelopoietic suppressant drugs. The

dose should be reduced by 50% if the serum bilirubin lies between 1.2 and 3 mg/dL and by 75% if above 3 mg/dL. The lifetime total dose should not exceed 550 mg/m² in patients with normal heart function and 400 mg/m² in persons having received mediastinal irradiation. Alternatively, 30 mg/m² on each of 3 consecutive days, repeated every 4 wk. Exemplary doses may be 10 mg/m², 20 mg/m², 30 mg/m², 50 mg/m², 100 mg/m², 150 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m², 275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

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ii. Daunorubicin

Daunorubicin hydrochloride, 5,12-Naphthacenedione, (8S-cis)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-a-L-lyxo-hexanopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-10-methoxy-, hydrochloride; also termed cerubidine and available from Wyeth. Daunorubicin intercalates into DNA, blocks DAN-directed RNA polymerase and inhibits DNA synthesis. It can prevent cell division in doses that do not interfere with nucleic acid synthesis.

In combination with other drugs it is included in the first-choice chemotherapy of acute myelocytic leukemia in adults (for induction of remission), acute lymphocytic leukemia and the acute phase of chronic myelocytic leukemia. Oral absorption is poor, and it must be given intravenously. The half-life of distribution is 45 minutes and of elimination, about 19 hr. The half-life of its active metabolite, daunorubicinol, is about 27 hr. Daunorubicin is metabolized mostly in the liver and also secreted into the bile (ca 40%). Dosage must be reduced in liver or renal insufficiencies.

Suitable doses are (base equivalent), intravenous adult, younger than 60 yr. 45 mg/m²/day (30 mg/m² for patients older than 60 yr.) for 1, 2 or 3 days every 3 or 4 wk or 0.8 mg/kg/day for 3 to 6 days every 3 or 4 wk; no more than 550 mg/m² should be given in a lifetime, except only 450 mg/m² if there has been chest irradiation; children, 25

mg/m² once a week unless the age is less than 2 yr. or the body surface less than 0.5 m, in which case the weight-based adult schedule is used. It is available in injectable dosage forms (base equivalent) 20 mg (as the base equivalent to 21.4 mg of the hydrochloride). Exemplary doses may be 10 mg/m², 20 mg/m², 30 mg/m², 50 mg/m², 100 mg/m², 150 5 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m², 275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

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iii. Mitomycin

Mitomycin (also known as mutamycin and/or mitomycin-C) is an antibiotic isolated from the broth of *Streptomyces caespitosus* which has been shown to have antitumor activity. The compound is heat stable, has a high melting point, and is freely soluble in organic solvents.

15

Mitomycin selectively inhibits the synthesis of deoxyribonucleic acid (DNA). The guanine and cytosine content correlates with the degree of mitomycin-induced cross-linking. At high concentrations of the drug, cellular RNA and protein synthesis are also suppressed.

20

In humans, mitomycin is rapidly cleared from the serum after intravenous administration. Time required to reduce the serum concentration by 50% after a 30 mg. bolus injection is 17 minutes. After injection of 30 mg, 20 mg, or 10 mg I.V., the maximal serum concentrations were 2.4 mg/mL, 1.7 mg/mL, and 0.52 mg/mL, respectively. Clearance is effected primarily by metabolism in the liver, but metabolism occurs in other tissues as well. The rate of clearance is inversely proportional to the maximal serum concentration because, it is thought, of saturation of the degradative pathways. Approximately 10% of a dose of mitomycin is excreted unchanged in the urine. Since metabolic pathways are saturated at relatively low doses, the percent of a 25

dose excreted in urine increases with increasing dose. In children, excretion of intravenously administered mitomycin is similar.

iv. Actinomycin D

5 Actinomycin D (Dactinomycin) [50-76-0]; $C_{62}H_{86}N_{12}O_{16}$ (1255.43) is an antineoplastic drug that inhibits DNA-dependent RNA polymerase. It is a component of first-choice combinations for treatment of choriocarcinoma, embryonal rhabdomyosarcoma, testicular tumor and Wilms' tumor. Tumors that fail to respond to systemic treatment sometimes respond to local perfusion. Dactinomycin potentiates
10 radiotherapy. It is a secondary (efferent) immunosuppressive.

Actinomycin D is used in combination with primary surgery, radiotherapy, and other drugs, particularly vincristine and cyclophosphamide. Antineoplastic activity has also been noted in Ewing's tumor, Kaposi's sarcoma, and soft-tissue sarcomas.
15 Dactinomycin can be effective in women with advanced cases of choriocarcinoma. It also produces consistent responses in combination with chlorambucil and methotrexate in patients with metastatic testicular carcinomas. A response may sometimes be observed in patients with Hodgkin's disease and non-Hodgkin's lymphomas. Dactinomycin has also been used to inhibit immunological responses, particularly the rejection of renal
20 transplants.

Half of the dose is excreted intact into the bile and 10% into the urine; the half-life is about 36 hr. The drug does not pass the blood-brain barrier. Actinomycin D is supplied as a lyophilized powder (0.5 mg in each vial). The usual daily dose is 10 to 15 mg/kg; this is given intravenously for 5 days; if no manifestations of toxicity are encountered, additional courses may be given at intervals of 3 to 4 weeks. Daily injections of 100 to 400 mg have been given to children for 10 to 14 days; in other regimens, 3 to 6 mg/kg, for a total of 125 mg/kg, and weekly maintenance doses of 7.5 mg/kg have been used. Although it is safer to administer the drug into the tubing of an
25 intravenous infusion, direct intravenous injections have been given, with the precaution
30 of

of discarding the needle used to withdraw the drug from the vial in order to avoid subcutaneous reaction. Exemplary doses may be 100 mg/m², 150 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m², 275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

5 v. Bleomycin

Bleomycin is a mixture of cytotoxic glycopeptide antibiotics isolated from a strain 10 of *Streptomyces verticillus*. Although the exact mechanism of action of bleomycin is unknown, available evidence would seem to indicate that the main mode of action is the inhibition of DNA synthesis with some evidence of lesser inhibition of RNA and protein synthesis.

15 In mice, high concentrations of bleomycin are found in the skin, lungs, kidneys, peritoneum, and lymphatics. Tumor cells of the skin and lungs have been found to have high concentrations of bleomycin in contrast to the low concentrations found in hematopoietic tissue. The low concentrations of bleomycin found in bone marrow may be related to high levels of bleomycin degradative enzymes found in that tissue.

20 In patients with a creatinine clearance of >35 mL per minute, the serum or plasma terminal elimination half-life of bleomycin is approximately 115 minutes. In patients with a creatinine clearance of <35 mL per minute, the plasma or serum terminal elimination half-life increases exponentially as the creatinine clearance decreases. In 25 humans, 60% to 70% of an administered dose is recovered in the urine as active bleomycin. Bleomycin may be given by the intramuscular, intravenous, or subcutaneous routes. It is freely soluble in water.

30 Bleomycin should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven

combinations with other approved chemotherapeutic agents in squamous cell carcinoma such as head and neck (including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, larynx), skin; penis, cervix, and vulva. It has also been used in the treatment of lymphomas and testicular carcinoma.

5

Because of the possibility of an anaphylactoid reaction, lymphoma patients should be treated with two units or less for the first two doses. If no acute reaction occurs, then the regular dosage schedule may be followed.

10

Improvement of Hodgkin's Disease and testicular tumors is prompt and noted within 2 weeks. If no improvement is seen by this time, improvement is unlikely. Squamous cell cancers respond more slowly, sometimes requiring as long as 3 weeks before any improvement is noted.

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d. Corticosteroid Hormones

Corticosteroid hormones are useful in treating some types of cancer (lymphoma, leukemias, and multiple myeloma). Though these hormones have been used in the treatment of many non-cancer conditions, they are considered chemotherapy drugs when they are implemented to kill or slow the growth of cancer cells. Corticosteroid hormones can increase the effectiveness of other chemotherapy agents, and consequently, they are frequently used in combination treatments. Prednisone and dexamethasone are examples of corticosteroid hormones.

25

e. Mitotic Inhibitors

Mitotic inhibitors include plant alkaloids and other natural agents that can inhibit either protein synthesis required for cell division or mitosis. They operate during a specific phase during the cell cycle. Mitotic inhibitors comprise docetaxel, etoposide (VP16), paclitaxel, taxol, vinblastine, vincristine, and vinorelbine.

i. Etoposide (VP16)

VP16 is also known as etoposide and is used primarily for treatment of testicular tumors, in combination with bleomycin and cisplatin, and in combination with cisplatin for small-cell carcinoma of the lung. It is also active against non-Hodgkin's lymphomas, 5 acute nonlymphocytic leukemia, carcinoma of the breast, and Kaposi's sarcoma associated with acquired immunodeficiency syndrome (AIDS).

VP16 is available as a solution (20 mg/ml) for intravenous administration and as 50-mg, liquid-filled capsules for oral use. For small-cell carcinoma of the lung, the 10 intravenous dose (in combination therapy) is can be as much as 100 mg/m² or as little as 2 mg/ m², routinely 35 mg/m², daily for 4 days, to 50 mg/m², daily for 5 days have also been used. When given orally, the dose should be doubled. Hence the doses for small 15 cell lung carcinoma may be as high as 200-250mg/m². The intravenous dose for testicular cancer (in combination therapy) is 50 to 100 mg/m² daily for 5 days, or 100 mg/m² on alternate days, for three doses. Cycles of therapy are usually repeated every 3 to 4 weeks. The drug should be administered slowly during a 30- to 60-minute infusion in order to avoid hypotension and bronchospasm, which are probably due to the solvents used in the formulation.

20 **ii. Taxol**

Taxol is an experimental antimitotic agent, isolated from the bark of the ash tree, *Taxus brevifolia*. It binds to tubulin (at a site distinct from that used by the vinca alkaloids) and promotes the assembly of microtubules. Taxol is currently being evaluated clinically; it has activity against malignant melanoma and carcinoma of the ovary. 25 Maximal doses are 30 mg/m² per day for 5 days or 210 to 250 mg/m² given once every 3 weeks. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

iii. Vinblastine

Vinblastine is another example of a plant alkaloid that can be used in combination with gene therapy for the treatment of cancer and precancer. When cells are incubated with vinblastine, dissolution of the microtubules occurs.

5

Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM. Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and 10 erythrocytes.

After intravenous injection, vinblastine has a multiphasic pattern of clearance from the plasma; after distribution, drug disappears from plasma with half-lives of approximately 1 and 20 hours. Vinblastine is metabolized in the liver to biologically 15 activate derivative desacetylvinblastine. Approximately 15% of an administered dose is detected intact in the urine, and about 10% is recovered in the feces after biliary excretion. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

20

Vinblastine sulfate is available in preparations for injection. The drug is given intravenously; special precautions must be taken against subcutaneous extravasation, since this may cause painful irritation and ulceration. The drug should not be injected into an extremity with impaired circulation. After a single dose of 0.3 mg/kg of body 25 weight, myelosuppression reaches its maximum in 7 to 10 days. If a moderate level of leukopenia (approximately 3000 cells/mm³) is not attained, the weekly dose may be increased gradually by increments of 0.05 mg/kg of body weight. In regimens designed to cure testicular cancer, vinblastine is used in doses of 0.3 mg/kg every 3 weeks irrespective of blood cell counts or toxicity.

30

The most important clinical use of vinblastine is with bleomycin and cisplatin in the curative therapy of metastatic testicular tumors. Beneficial responses have been reported in various lymphomas, particularly Hodgkin's disease, where significant improvement may be noted in 50 to 90% of cases. The effectiveness of vinblastine in a high proportion of lymphomas is not diminished when the disease is refractory to alkylating agents. It is also active in Kaposi's sarcoma, neuroblastoma, and Letterer-Siwe disease (histiocytosis X), as well as in carcinoma of the breast and choriocarcinoma in women.

Doses of vinblastine will be determined by the clinician according to the individual patients need. 0.1 to 0.3 mg/kg can be administered or 1.5 to 2mg/m² can also be administered. Alternatively, 0.1 mg/m², 0.12 mg/m², 0.14 mg/m², 0.15 mg/m², 0.2 mg/m², 0.25 mg/m², 0.5 mg/m², 1.0 mg/m², 1.2 mg/m², 1.4 mg/m², 1.5 mg/m², 2.0 mg/m², 2.5 mg/m², 5.0 mg/m², 6 mg/m², 8 mg/m², 9 mg/m², 10 mg/m², 20 mg/m², can be given. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

iv. Vincristine

Vincristine blocks mitosis and produces metaphase arrest. It seems likely that most of the biological activities of this drug can be explained by its ability to bind specifically to tubulin and to block the ability of protein to polymerize into microtubules. Through disruption of the microtubules of the mitotic apparatus, cell division is arrested in metaphase. The inability to segregate chromosomes correctly during mitosis presumably leads to cell death.

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The relatively low toxicity of vincristine for normal marrow cells and epithelial cells make this agent unusual among anti-neoplastic drugs, and it is often included in combination with other myelosuppressive agents.

Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM.

5 Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes. Vincristine has a multiphasic pattern of clearance from the plasma; the terminal half-life is about 24 hours. The drug is metabolized in the liver, but no biologically active derivatives have been identified. Doses should be reduced in patients with hepatic dysfunction. At least a
10 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Vincristine sulfate is available as a solution (1 mg/ml) for intravenous injection. Vincristine used together with corticosteroids is presently the treatment of choice to induce remissions in childhood leukemia; the optimal dosages for these drugs appear to be vincristine, intravenously, 2 mg/m^2 of body-surface area, weekly, and prednisone, orally, 40 mg/m^2 , daily. Adult patients with Hodgkin's disease or non-Hodgkin's lymphomas usually receive vincristine as a part of a complex protocol. When used in the MOPP regimen, the recommended dose of vincristine is 1.4 mg/m^2 . High doses of vincristine seem to be tolerated better by children with leukemia than by adults, who may experience severe neurological toxicity. Administration of the drug more frequently than every 7 days or at higher doses seems to increase the toxic manifestations without proportional improvement in the response rate. Precautions should also be used to avoid extravasation during intravenous administration of vincristine. Vincristine (and vinblastine) can be infused into the arterial blood supply of tumors in doses several times larger than those that can be administered intravenously with comparable toxicity.

Vincristine has been effective in Hodgkin's disease and other lymphomas. Although it appears to be somewhat less beneficial than vinblastine when used alone in
30 Hodgkin's disease, when used with mechlorethamine, prednisone, and procarbazine (the

so-called MOPP regimen), it is the preferred treatment for the advanced stages (III and IV) of this disease. In non-Hodgkin's lymphomas, vincristine is an important agent, particularly when used with cyclophosphamide, bleomycin, doxorubicin, and prednisone. Vincristine is more useful than vinblastine in lymphocytic leukemia. Beneficial response 5 have been reported in patients with a variety of other neoplasms, particularly Wilms' tumor, neuroblastoma, brain tumors, rhabdomyosarcoma, and carcinomas of the breast, bladder, and the male and female reproductive systems.

Doses of vincristine for use will be determined by the clinician according to the 10 individual patients need. 0.01 to 0.03mg/kg or 0.4 to 1.4mg/m² can be administered or 1.5 to 2mg/m² can also be administered. Alternatively 0.02 mg/m², 0.05 mg/m², 0.06 mg/m², 0.07 mg/m², 0.08 mg/m², 0.1 mg/m², 0.12 mg/m², 0.14 mg/m², 0.15 mg/m², 0.2 mg/m², 0.25mg/m² can be given as a constant intravenous infusion. Of course, all of these 15 dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

f. Nitrosoureas

Nitrosoureas, like alkylating agents, inhibit DNA repair proteins. They are used to 20 treat non-Hodgkin's lymphomas, multiple myeloma, malignant melanoma, in addition to brain tumors. Examples include carmustine and lomustine.

a. Carmustine

Carmustine (sterile carmustine) is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1,3bis (2-chloroethyl)-1-nitrosourea. It is lyophilized 25 pale yellow flakes or congealed mass with a molecular weight of 214.06. It is highly soluble in alcohol and lipids, and poorly soluble in water. Carmustine is administered by intravenous infusion after reconstitution as recommended

Although it is generally agreed that carmustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

5 Carmustine is indicated as palliative therapy as a single agent or in established combination therapy with other approved chemotherapeutic agents in brain tumors such as glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors. Also it has been used in combination with prednisone to treat multiple myeloma. Carmustine has proved useful, in the treatment of Hodgkin's Disease
10 and in non-Hodgkin's lymphomas, as secondary therapy in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

15 Sterile carmustine is commonly available in 100 mg single dose vials of lyophilized material. The recommended dose of carmustine as a single agent in previously untreated patients is 150 to 200 mg/m² intravenously every 6 weeks. This may be given as a single dose or divided into daily injections such as 75 to 100 mg/m² on 2 successive days. When carmustine is used in combination with other myelosuppressive drugs or in patients in whom bone marrow reserve is depleted, the doses should be
20 adjusted accordingly. Doses subsequent to the initial dose should be adjusted according to the hematologic response of the patient to the preceding dose. It is of course understood that other doses may be used in the present invention for example 10mg/m², 20mg/m², 30mg/m² 40mg/m² 50mg/m² 60mg/m² 70mg/m² 80mg/m² 90mg/m² 100mg/m².
25 The skilled artisan is directed to, "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

ii. Lomustine

Lomustine is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1-(2-chloro-ethyl)-3-cyclohexyl-1 nitrosourea. It is a yellow powder with the empirical formula of $C_9H_{16}ClN_3O_2$ and a molecular weight of 233.71. Lomustine is soluble in 10% ethanol (0.05 mg per mL) and in absolute alcohol (70 mg per mL). Lomustine is relatively insoluble in water (<0.05 mg per mL). It is relatively unionized at a physiological pH. Inactive ingredients in lomustine capsules are: magnesium stearate and mannitol.

10 Although it is generally agreed that lomustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

15 Lomustine may be given orally. Following oral administration of radioactive lomustine at doses ranging from 30 mg/m^2 to 100 mg/m^2 , about half of the radioactivity given was excreted in the form of degradation products within 24 hours. The serum half-life of the metabolites ranges from 16 hours to 2 days. Tissue levels are comparable to plasma levels at 15 minutes after intravenous administration.

20 Lomustine has been shown to be useful as a single agent in addition to other treatment modalities, or in established combination therapy with other approved chemotherapeutic agents in both primary and metastatic brain tumors, in patients who have already received appropriate surgical and/or radiotherapeutic procedures. It has also proved effective in secondary therapy against Hodgkin's Disease in combination with other approved drugs in patients who relapse while being treated with primary therapy, or 25 who fail to respond to primary therapy.

30 The recommended dose of lomustine in adults and children as a single agent in previously untreated patients is 130 mg/m^2 as a single oral dose every 6 weeks. In individuals with compromised bone marrow function, the dose should be reduced to 100

mg/m² every 6 weeks. When lomustine is used in combination with other myelosuppressive drugs, the doses should be adjusted accordingly. It is understood that other doses may be used for example, 20mg/m² 30mg/m², 40 mg/m², 50mg/m², 60mg/m², 70mg/m², 80mg/m², 90mg/m², 100mg/m², 120mg/m² or any doses between these figures
5 as determined by the clinician to be necessary for the individual being treated.

g. **Miscellaneous Agents**

Some chemotherapy agents do not qualify into the previous categories based on their activities. However, it is contemplated that they are included within the method of
10 the present invention for use in combination therapies of cancer with gene therapy involving lipid formulations. They include amsacrine, L-asparaginase, tretinoin, and Tumor Necrosis Factor (TNF), some of which are discussed below.

i. **Tumor Necrosis Factor**

15 Tumor Necrosis Factor (TNF; Cachectin) is a glycoprotein that kills some kinds of cancer cells, activates cytokine production, activates macrophages and endothelial cells, promotes the production of collagen and collagenases, is an inflammatory mediator and also a mediator of septic shock, and promotes catabolism, fever and sleep. Some infectious agents cause tumor regression through the stimulation of TNF production.
20 TNF can be quite toxic when used alone in effective doses, so that the optimal regimens probably will use it in lower doses in combination with other drugs. Its immunosuppressive actions are potentiated by gamma-interferon, so that the combination potentially is dangerous. A hybrid of TNF and interferon- α also has been found to possess anti-cancer activity.

25

3. Radiotherapy

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad
30

range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, 5 and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic construct and a chemotherapeutic or 10 radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

4. Immunotherapy

15 Immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, 20 radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve merely as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells. Mda-7 gene transfer to tumor cells causes tumor cell death and apoptosis. The apoptotic tumor cells are scavenged by 25 reticuloendothelial cells including dendritic cells and macrophages and presented to the immune system to generate anti-tumor immunity (Rovere *et al.*, 1999; Steinman *et al.*, 1999). The soluble form of MDA-7 protein has cytokine-like structure and activities, such as activation of immune cells. The combination of therapeutic modalities, i.e., direct cytotoxic activity and immune activation by MDA-7 would provide therapeutic 30 benefit in the treatment of cancer.

Immunotherapy could also be used as part of a combined therapy, in conjunction with Ad-md₄ gene therapy. The general approach for combined therapy is discussed below. In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, erb B and p155. An alternative aspect of immunotherapy is to combine pro-apoptotic effect, mediated by Ad-md₄ treatment with immune stimulatory effects. The latter may be inherent in the soluble MDA-7 protein. However, alternate immune stimulating molecules also exist including: cytokines such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines such as MIP-1, MCP-1, IL-8 and growth factors such as FLT3 ligand. Combining immune stimulating molecules, either as proteins or using gene delivery in combination with Ad-md₄ will enhance anti-tumor effects (Ju *et al.*, 2000).

a. Passive Immunotherapy

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been administered intralesionally. Human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie & Morton, 1986). Regression was observed in six out of ten patients, following,

daily or weekly, intralesional injections. In another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

5 It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune enhancers as described by Bajorin *et al.* (1988). The development of human monoclonal antibodies is described in further detail elsewhere in the specification.

10 **b. Active Immunotherapy**

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath & Morton, 1991; Morton & 15 Ravindranath, 1996; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992). IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or ant(carbohydrate) antibodies.

20 **c. Adoptive Immunotherapy**

In adoptive immunotherapy, the patient's circulating lymphocytes, or tumor infiltrated lymphocytes, are isolated *in vitro*, activated by lymphokines such as IL-2 or transduced with genes for tumor necrosis, and readministered (Rosenberg *et al.*, 1988; 25 1989). To achieve this, one would administer to an animal, or human patient, an immunologically effective amount of activated lymphocytes in combination with an adjuvant-incorporated antigenic peptide composition as described herein. The activated lymphocytes will most preferably be the patient's own cells that were earlier isolated from a blood or tumor sample and activated (or "expanded") *in vitro*. This form of 30 immunotherapy has produced several cases of regression of melanoma and renal

carcinoma, but the percentage of responders were few compared to those who did not respond.

5. Surgery

5 Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative and palliative surgery. Curative surgery is a cancer treatment that may be used in conjunction with other therapies, such as the treatment of the present invention, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy and/or alternative therapies.

10

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). It is further contemplated that the present invention may be used in conjunction with removal of superficial cancers, precancers, or incidental amounts of normal tissue.

20 Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

25

6. Other Anti-Cancer Therapies

It is contemplated that other therapies and agents may be used in combination with the present invention to improve the therapeutic efficacy of treatment. These additional agents include immunomodulatory agents, agents that affect the upregulation 30 of cell surface receptors and GAP junctions, cytostatic and differentiation agents,

inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Immunomodulatory agents include tumor necrosis factor; interferon alpha, beta, and gamma; IL-2 and other cytokines; F42K and other cytokine analogs; or MIP-1, MIP-1beta, MCP-1, RANTES, and other chemokines. It is further contemplated that the upregulation of cell surface receptors or their ligands such as Fas / Fas ligand, DR4 or DR5 / TRAIL (Apo-2 ligand) would potentiate the apoptotic inducing abilities of the present invention by establishment of an autocrine or paracrine effect on hyperproliferative cells. Increases intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with the present invention to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present invention. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with the present invention to improve the treatment efficacy.

Another form of therapy for use in conjunction with chemotherapy, radiation therapy or biological therapy includes hyperthermia, which is a procedure in which a patient's tissue is exposed to high temperatures (up to 106°F). External or internal heating devices may be involved in the application of local, regional, or whole-body hyperthermia. Local hyperthermia involves the application of heat to a small area, such as a tumor. Heat may be generated externally with high-frequency waves targeting a tumor from a device outside the body. Internal heat may involve a sterile probe, including thin, heated wires or hollow tubes filled with warm water, implanted microwave antennae, or radiofrequency electrodes.

A patient's organ or a limb is heated for regional therapy, which is accomplished using devices that produce high energy, such as magnets. Alternatively, some of the

patient's blood may be removed and heated before being perfused into an area that will be internally heated. Whole-body heating may also be implemented in cases where cancer has spread throughout the body. Warm-water blankets, hot wax, inductive coils, and thermal chambers may be used for this purpose.

5

Hormonal therapy may also be used in conjunction with the present invention or in combination with any other cancer therapy previously described. The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as 10 testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

G. EXAMPLES

The following examples are included to demonstrate preferred embodiments of 15 the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific 20 embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1**MATERIALS AND METHODS**

5 **Animals.** 3-6 wk-old female/male BALB/c nude and Beige/SCID mice were purchased from Harlan Inc. (Indianapolis, IN) and Charles River laboratories, respectively. Animals were housed in specific pathogen-free units of the Department of Veterinary Medicine and Surgery at M. D. Anderson Cancer Center.

10 **Preparation of lipid formulation.** DOTAP:Cholesterol liposomes were prepared by the methods of Templeton *et al.* (1997). Briefly, DOTAP (cationic lipid) was mixed with cholesterol (neutral lipid) at equimolar concentrations. This mixture of powdered lipids was then dissolved with HPLC grade chloroform (Mallinckrodt) in a 1L round bottom flask. The lipid solution was dried to a thin film at 30°C for 30 minutes using a Buchi rotary evaporator. The flask containing the thin film was further dried under 15 vacuum for 15 minutes. The film was hydrated in water containing 5% dextrose (w/v) to give a final concentration of 20 mM DOTAP and 20 mM cholesterol. The hydrated lipid film was rotated in a 50°C water bath for 45 minutes and then at 35°C for an additional 10 minutes. The mixture was left standing at room temperature overnight. The following day the mixture was sonicated for 5 minutes at 50°C. The sonicated mixture was 20 transferred to a tube and was heated for 10 minutes at 50°C. This mixture was sequentially extruded through Whatman syringe filters of decreasing pore size (1 µm, 0.45 µm, 0.2 µm, 0.1 µm). The 0.2 µm and 0.1 µm were Whatman Anotop filters. Filtrate was stored at 4°C under argon gas.

25 **Lipid complex preparation.** Lipid complex (DNA : lipid complex) were prepared on the day of application. DNA and lipids were diluted in 5% dextrose in water to obtain an appropriate concentration of DNA and lipids. Equal volumes of DNA and lipids, at a concentration to obtain 100 µg of DNA / 5mM lipids / 100 µl, were mixed by

adding the DNA rapidly to the surface of the lipid solution followed by two rapid up and down expulsions from a Pipetman.

5 **Lipid complex characterization.** Lipid complexes were produced as previously described and electrophoresed at 60-80 V/cm² through a 0.8% agarose gel using 1X TBE as running buffer at room temperature for 1-2 hours. DNA is visible as an ethidium bromide stained band characteristic of uncut source plasmid. DNA:lipid is not visible with this technique due to the size of the complex and its inability to enter the agarose gel. The mean particle size was determined by dynamic light scattering using a Coulter 10 N4 particle size analyzer. The average particle size is 310-320 nm. The various DNAs did not alter the particle size of the lipid complex.

15 **Reagents and cell lines.** DOTAP was purchased from Avanti Polar Lipids. Cholesterol (high purity) was purchased from Calbiochem. H1299, A549, H322, and H226Br tumor cell lines were a gift from Dr. Adi Gazdar and Dr. John Minna, University of Texas Southwestern Medical Center, Dallas, Tx.

20 **Cell preparation. *In vitro* transfection assay.** Cells were plated at a density of 5x10⁵ cells per 60 mm² in RPMI /10% FBS media and grown in 5% CO₂ at 37°C.

25 **Tail vein injections.** Cells were plated at an approximately 20-40% confluency in RPMI /10% FBS media, supplemented with penicillin, streptomycin and fungizone, and grown in 5% CO₂ at 37°C until approximately 80% confluent. A549 cells were grown and maintained in Ham's F12 instead of RPMI. Cells were washed twice in PBS, trypsinized, and counted. Cells were diluted to a concentration of 1x10⁶ cells / 200 µl in PBS.

Subcutaneous injections. Cells were plated at a density of approximately 20-40% confluency in 150 mm² dishes in RPMI /10% FBS media and grown in 5% CO₂ at

37°C until approximately 80% confluent. Cells were washed twice in PBS, trypsinized, and counted. Cells were diluted to a concentration of 5×10^6 cells / 100 μ l in PBS.

5 **Solid tumor induction by subcutaneous injection.** BALB/c nude mice were injected subcutaneously with 5×10^6 tumor cells in 100 μ l of PBS.

10 **Lung tumor induction by tail vein injection.** Beige SCID mice were injected via the tail vein with 1×10^6 tumor cells in 200 μ l of PBS using a 27 gauge syringe needle.

15 **Analysis of p53 protein expression.** Expression of p53 was detected by western blotting of transfected cells. Expression of p53 *in vivo* was determined by immunohistochemical staining of tumor sections.

EXAMPLE 2

15 ***IN VITRO* LIPID COMPLEX GENE DELIVERY TO TUMOR CELL LINES**

20 *In vitro* transfection studies were used to show lipid complex gene transfer into the tumor cell lines utilized in subsequent studies. Lipid : nucleic acid complex containing an expression construct encoding green fluorescent protein (GFP, lipid complex-GFP) was used as a reporter construct. Efficiencies of gene transfer were studied in the tumor cell lines H1299, H322, H226Br, and A549. Cell lines were plated as previously described and grown overnight. The following day, cells were transfected with 5 μ l of lipid complex-GFP (50 μ g / 100 μ l of lipid complex-GFP) and grown in serum free media for 3 hours at 37°C in 5% CO₂. After a 3 hour incubation, serum free media was aspirated and replaced with complete media. Cells were grown overnight at 37°C in 5% CO₂. Cells were harvested on the following day and washed twice with PBS. Cells were resuspended in a final volume of 300 μ l PBS. GFP fluorescence was quantitated using flow assisted cell sorting (FACS) analysis (Coulter Corporation).

EXAMPLE 3

IN VIVO LIPID COMPLEX GENE DELIVERY

Lipid complex- β gal was administered by tail vein injection into 3-4 week old female BALB/c nude mice. Lipid complex gene transfer studies to the lung were conducted by standard histochemical and immuno-histochemical staining for β gal expression. BALB/c nude mice were injected with (1) Ad- β gal at 10^9 pfu, (2) β gal DNA 100 μ g/200 μ l volume, and (3) lipid complex- β gal 100 μ g/5mM lipids in a 200 μ l volume. Mice were killed 48 hours post injection and exsanguinated. Lung tissue from each experimental group was harvested and mounted in O.C.T. compound for cryosections. Tissues mounted in O.C.T. were cut into 4-6 μ m thick sections. The cryosections were mounted on silane coated slides, air dried at room temperature, and stained histochemically for LacZ expression using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) substrate (Sigma) as previously described (Turner *et al.* 1990). After rinsing with water for 5 minutes, sections were counterstained with Meyer's hematoxylin (Sigma) or neutral red (Fisher), dehydrated in ethanol, immersed for 5 minutes in xylene, and then mounted under coverslips. Control animals not treated were included to assure specificity of the staining.

Cells positive and negative for lacZ activity were counted under a microscope at a magnification of 200x in a double blind fashion. Ad- β gal treated animals had an increase in positive cells of 4% over background. Lipid complex- β gal treated animals had an increase in positive cells of 11% over background. It can be concluded that *in vivo* administration of lipid complex- β gal transfects lung cells with a higher efficiency than does Ad- β gal. These data, as well previously published data, confirm the utility of lipid complexes for nucleic acid delivery to the lung.

EXAMPLE 4***IN VIVO LIPID COMPLEX-P53 GENE DELIVERY TO H1299 SUBCUTANEOUS TUMORS***

5 For *in vivo* delivery of lipid complex-P53 3-4 week old female BALB/c nude mice were subcutaneously injected with 5×10^6 H1299 tumor cells per animal. Animals were treated five days post-H1299 injection by intratumoral injection of 200 μl of lipid complex-P53 (100 μg DNA / 5mM lipid). Tumors were measured every other day. Treatment groups were 1) no treatment 2) intratumoral injection every day for 6 days
10 with lipid complex-P53 DNA (100 μg in a 200 μl volume) 3) intratumoral injection every day for 6 days with P53 DNA (100 μg in a 200 μl volume). Tumor size was measured every other day for 16 days. Lipid complex-P53 DNA showed a significant inhibition of tumor growth compared to no treatment and DNA alone.

15

EXAMPLE 5***INTRAVENOUS LIPID COMPLEX-P53 GENE DELIVERY TO H1299 PULMONARY METASTASES***

A lung tumor model was established by injection of 1×10^6 H1299 tumor cells in
20 200 μl of PBS via the tail vein of Beige/SCID mice. Three days post injection mice were administered 100 μl of lipid complex-p53 (50 μg DNA / 5 mM lipids) via tail vein injection. Treatments were continued every other day for a total of 6 treatments. Animals were killed on day 21 and injected with India ink intratracheally. Pulmonary tumor nodules were visualized by developing the lungs in Feketes solution and were
25 counted by visual inspection with the aid of a dissecting microscope. Control animals displayed approximately 400 pulmonary nodules. The lipid complex-p53 treated animals displayed approximately 10 pulmonary nodules per animal. The administration of lipid complex-P53 via tail vein injection significantly reduced tumor formation in the lungs of Beige/SCID mice.

EXAMPLE 6**INTRAVENOUS LIPID COMPLEX-P53 GENE DELIVERY AND P53 GENE EXPRESSION
ANALYSIS IN H1299 SUBCUTANEOUS TUMORS**

5

A subcutaneous tumor model was established by injection of 5×10^6 H1299 tumor cells in 100 μ l of PBS subcutaneously in BALB/c nude mice. Mice were administered 100 μ l of lipid complex-p53 (50 μ g DNA) via tail vein 5 days post subcutaneous tumor cell injection. Animals were killed 24 hours post-treatment and tissues harvested. Lung, 10 spleen, and liver tissues were harvested and fixed in formalin. Tissue sections were analyzed using standard immuno-histochemical staining for the P53 protein. Immunostaining detected significant levels of P53 protein expression within tumor cells. No treatment and p53 DNA alone resulted in no detectable P53 protein.

15

EXAMPLE 7**TARGETING ENHANCED LIPID COMPLEX -P53 DELIVERY TO TUMOR CELLS
RECALCITRANT TO TRANSFECTION BY LIPID COMPLEX P53**

20

Although H1299 cells were readily transfected *in vitro* with lipid complex- β gal, other cell lines were resistant. Tumor cell lines H322, H226Br, and A549 have been previously shown to exhibit low lipid complex transfection frequencies. In an attempt to enhance the transfection of resistant cell lines, antibody or cyclic peptide targeting moieties were associated with the lipid complex- β gal. The antibody targeting moiety is a monoclonal anti-EGF receptor antibody. The peptide targeting moiety is a cyclic peptide containing within its sequence a RGD integrin binding motif. These targeting moieties were non-covalently associated with the lipid complex. It is contemplated that a covalent attachment of the targeting moiety will further enhance the targeting efficiency of the lipid complex. Lipid complexes with associated targeting moieties demonstrated an enhanced transfection of A549 cells *in vitro* compared to lipid complexes without targeting moieties.

EXAMPLE 8
MATERIALS AND METHODS

5 **Materials.** All lipids (DOTAP, DOPE, cholesterol) were purchased from Avanti
Polar Lipids (Albaster, AL). RPMI-1640 medium, Ham's/F12 medium and fetal bovine
serum (FBS) were purchased from GIBCO-BRL-Life Technologies (New York, NY).
10 Polyclonal rabbit antihuman *FHIT* antibody and mouse antihuman p53 monoclonal
antibody (BP53.12) were obtained from Zymed Laboratories (San Francisco, CA) and
Santa-Cruz Biotechnology, Inc. (Palo Alto, CA) respectively.

Cell lines and animals. Human non-small cell lung carcinoma cell lines, H1299
(p53^{null}/*FHIT*) and A549 (p53⁺/*FHIT*) were obtained from American Type Culture
Collection and maintained in RPMI-1640 and Hams-F12 medium supplemented with
15 10% FBS, 1% Glutamate and antibiotics. Murine fibrosarcoma cell line, 2337m, which is
mutant for murine p53 was obtained from Dr. Isaiah J. Fidler, M.D. Anderson Cancer
Center and maintained in DMEM supplemented with 10% FBS. Cells were regularly
passaged and tested for presence of mycoplasma. 4-6 weeks old female C3H mice (NCI,
Frederick, MD), BALB/c nude (*nu/nu*) mice (Harlan-Sprague Dawley Inc., Indianapolis,
IN), and SCID/Beige mice (Charles River Laboratories, Wilmington, MA) used in the
20 study were maintained in a pathogen free environment and handled according to
institutional guidelines established for animal care and use.

Synthesis of liposomes and preparation of DNA:liposome mixture.
25 Liposomes (20 mM DOTAP:Chol, and 20 mM DOTAP:DOPE) were synthesized and
extruded through Whatman filters (Kent, UK) of decreasing size (1.0, 0.45, 0.2, and 0.1
μm) as described previously (Templeton, 1997). The synthesized liposomes were stored
under argon gas at 4°C. DNA:liposome complexes were prepared fresh two to three
hours prior to tail vein injection in mice. Briefly, DOTAP:Chol (20 mM) or
30 DOTAP:DOPE (20 mM) stock solution and stock DNA solution diluted in 5% dextrose

in water (D5W) were mixed in equal volumes to give a final concentration of 4 mM DOTAP:Chol-150 µg DNA in 300 µl final volume (ratio 1:2.6) ("Chol" refers to Cholesterol). Dilution and mixing of all reagents were at room temperature. Reagents were gently mixed in a 1.5 ml Eppendorf tube by pipetting. The DNA solution was 5 added at the surface of the liposome and mixed rapidly up and down twice with the pipet tip. The DNA:liposome mixture thus prepared was precipitate-free and used for all *in vivo* experiments.

Measurement of Particle Size Analysis. Freshly prepared DNA:liposome complexes were analyzed for mean particle size using the N4 particle size analyzer 10 (Coulter, Miami, FL). The average mean particle size of the DNA:liposome complexes ranged between 300-325nm.

15 *In vivo transfection efficiency in subcutaneous tumor, normal lung and tumor-bearing lungs.* Prior to the start of the experiment, *nu/nu* mice were subjected to 3.5Gy of total body irradiation using a cesium source according to institutional guidelines. Mice were then injected with *p53* gene-null H1299 tumor cells (5×10^6 /100 µl of PBS) subcutaneously on the right flank. When the tumors reached 4-5 mm³ in size, a single dose of DOTAP:Chol- DNA:liposome complex (100 µg of *Lac-Z* DNA) was 20 injected intratumorally. Forty-eight hours after injection, mice were euthanized by CO₂ inhalation, and tumors were removed and analyzed histochemically for β-galactosidase expression (Couffinhal, 1997). Tumors were cut into 4-µm-thick sections, stained for β-galactosidase, and evaluated by light microscopy.

25 *p53 and Fhit protein expression in tumors was determined by western blot analysis.* Briefly, subcutaneous H1299 tumors injected with DOTAP:Chol-DNA:liposome complex (100 µg of *Lac-Z*, *CAT*, *p53*, and *FHIT* DNA) were harvested at 48 hours and homogenized in Laemmeli buffer. Protein concentration was determined using Bio-Rad protein assay reagent (Bio-Rad, Fremont, CA) and 50 µg of total protein

was analyzed by SDS-PAGE electrophoresis. p53 and Fhit proteins were detected using mouse antihuman *p53* antibody (BP53.12) and rabbit antihuman *FHIT* antibody as described previously (Ji *et al.*, 1999; Hamada *et al.*, 1996; Sozzi *et al.*, 1997).

5 To determine the transduction efficiency in normal lungs, mice were injected with DOTAP:Chol-*Lac-Z* or *p53* DNA:liposome complexes intravenously (i.v.) via tail vein. Forty-eight hours post injection, animals were euthanized by CO₂ inhalation and lungs harvested and either snap frozen for β-galactosidase analysis or formalin fixed for p53 analysis. Tissue sections were cut and analyzed histochemically (β-gal) or 10 immunohistochemically (p53) as described previously (Couffinhal, 1997). To determine the transduction efficiency in lung tumors *in vivo*, nude mice were injected with 1x10⁶ A549 tumor cells suspended in 200 μl of PBS i.v. via tail vein. Two to three weeks later, a single dose of *Lac-Z* or *FHIT* DNA-DOTAP:Chol liposome complex (50 μg) or naked plasmid DNA (50 μg) was injected via tail vein. Lungs were harvested forty eight hours 15 later and analyzed for protein expression by histochemical (β-gal) or immunohistochemical (Fhit) analysis (Couffinhal, 1997; Sozzi *et al.*, 1997).

Evaluation of tumor growth and treatments *in vivo*. Prior to the start of all experiments involving subcutaneous tumor growth and treatments, *nu/nu* mice were 20 irradiated (3.5Gy) using a cesium source to enhance tumor uptake. In all the experiments, 5x10⁶ tumor cells (H1299, A549) suspended in 100 μl sterile phosphate buffered saline (PBS) were injected into the right dorsal flank. When the tumor had reached a size of 4-5mm³, animals were randomized into groups and treatment initiated. Intratumoral injections were performed under anesthesia using methoxyflurane (Schering 25 Plough, Kenilworth, NJ) as per institutional guidelines. Tumor measurements were recorded every other day without knowledge of the treatment groups, and the volume was calculated using the formula V (mm³) = a x b²/2, where "a" is the largest dimension and "b" is the perpendicular diameter (Georges *et al.*, 1993). Antitumor efficacy data are

presented as cumulative tumor volumes for all animals in each group to account for both size and number of tumors.

For p53 experiments, subcutaneous H1299 tumor bearing animals were divided
5 into three groups. Groups of eight animals were treated as follows: Group 1 received no treatment, Group 2 received naked *p53* plasmid DNA (100 µg/dose) and Group 3 received DOTAP:Chol-*p53* DNA:liposome complex (100 µg/dose) daily for a total of six doses. In a separate but identical experiment, an additional control group was included that received DOTAP:Chol-*pAd* DNA:liposome complex. All other experimental
10 conditions and treatment schedules were identical.

For *FHIT* experiments, H1299 and A549 subcutaneous tumors were established in nude mice. For each tumor type, four treatment groups were established comprised of seven animals per group. Treatment groups included: Group 1 received no treatment,
15 Group 2 received *FHIT* plasmid DNA (100 µg/dose), Group 3 received DOTAP:Chol-*CAT* DNA:liposome complex (100 µg/dose) and Group 4 received DOTAP:Chol-*FHIT* DNA: liposome complex (100 µg/dose). Animals were treated daily for a total of six doses. In all experiments, statistical differences in tumor size were determined using the Student's *t* test.
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Evaluation of lung metastases and treatments *in vivo*. To establish lung metastases, female SCID/Beige mice were injected intravenously via tail vein with 10⁶ H1299 tumor cells suspended in 200 µl of sterile PBS. Three days later, mice were divided into six groups and treated as follows: no treatment (Group 1), naked *p53* plasmid DNA (Group 2), DOTAP-DOPE-*p53* DNA:liposome complex (Group 3), DOTAP:Chol-*CAT* DNA:liposome complex (Group 4), non-extuded DOTAP:Chol-*p53* DNA:liposome complex (Group 5) and DOTAP:Chol-*p53* DNA:liposome complex (Group 6). There were eight mice in each group. Mice were treated with 50 µg of plasmid DNA or 50 µg DNA:liposome complex i.v. via tail vein using a 27 gauge needle daily for a total of six
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doses. Two weeks following the last dose, animals were euthanized by CO₂ inhalation. Lungs from each of the mice from the six groups were injected intra-tracheally with India ink and fixed in Feketes solution (Kataoka *et al.*, 1998). The therapeutic effect of systemic p53 gene treatment was determined by counting the number of metastatic tumors in each lung under a dissecting microscope without knowledge of the treatment groups. The data were analyzed and interpreted as statistically significant if the *p* value was <0.05 by the Mann-Whitney rank-sum test.

The A549 tumor model was used to determine the therapeutic effect of *p53* and *FHIT* tumor suppressor genes on *p53* wild type lung tumor cells. Mice (*nu/nu*) were injected with A549 tumor cells (1x10⁶) i.v via tail vein. On day 6, mice were divided into four groups (n=6 or 8 animals per group) and received the following treatment. Group 1 received no treatment, group 2 received *p53* or *FHIT* plasmid DNA, group 3 received extruded DOTAP:Chol-*CAT* DNA:liposome complex and group 4 received extruded DOTAP:Chol-*p53* DNA:liposome complex or DOTAP:Chol-*FHIT* DNA:liposome complex. Animals were treated daily for a total of six doses (50 µg/dose). Following the last dose, mice were euthanized and therapeutic effect of *p53* DNA:liposome and *FHIT* DNA:liposome complex treatments determined as described above for the H1299/SCID/Beige model.

Immunohistochemical Analysis. Subcutaneous H1299 tumors established in *nu/nu* mice that were either not treated or treated with *p53* plasmid DNA or treated with *p53* DNA:liposome complex were harvested and fixed in 4% buffered formalin, paraffin embedded and cut as 4 µm thick sections. Tissue sections were stained for *p53* gene expression as previously described (Fujiwara *et al.*, 1993; Fujiwara *et al.*, 1995). The number of tumor cells staining positive for *p53* were analyzed under bright field microscopy and quantitated without prior knowledge of the treatment groups. A total of at least five fields per specimen were analyzed. To determine the fate of tumor cells following treatment, subcutaneous tumors and tumor bearing lung sections were stained for apoptotic cell death using terminal deoxynucleotide transferase (Tdt) (Boehringer

Mannheim) and counterstained with methylene blue or methyl green as described previously (Fujiwara *et al.*, 1993; Fujiwara *et al.*, 1995). In all the staining procedures, appropriate negative controls were included.

5 **Histopathology.** To determine the therapeutic effect of the p53 gene on metastatic lung tumors, tumor bearing lungs were harvested from *nu/nu* mice at 21 days post treatment and evaluated histopathologically for tumor size, viability and mitotic index. Analysis was done by a pathologist without prior knowledge of the treatment groups.

10 **Survival Experiments.** To determine the efficacy of systemic treatment, survival experiments were performed using the two metastatic lung tumor (H1299, A549) models. Briefly, female SCID/Beige mice were injected with 10^6 H1299 tumor cells via the tail vein. Six days later, mice were divided into four groups of six mice each as follows:
15 Group 1 received no treatment, Group 2 received naked *p53* plasmid DNA, Group 3 received DOTAP:Chol-*CAT* DNA:liposome complex, and Group 4 received DOTAP:Chol-*p53* DNA:liposome complex. The treatment schedule consisted of six daily injections of naked plasmid DNA or DNA:liposome complex in 100 μ l volumes (50 μ g DNA/dose). Mice were monitored daily following the last injection. Moribund
20 animals were euthanized by CO₂ inhalation. Lungs, heart, liver, spleen, brain, kidney, colon, ovaries, pancreas, and bone from each animal were removed and analyzed histopathologically for the presence of disseminated tumors and for treatment associated toxicity. Statistical differences in actuarial survival curves were analyzed using the Kaplan-Meier survival estimation and Wilcoxon signed rank tests.

25 The A549 lung metastatic tumor model was used to evaluate the effect of the DOTAP:Chol-*p53* DNA:liposome complex on tumors that have the wild-type *p53* gene. Briefly, *nu/nu* mice were injected with 10^6 A549 tumor cells i.v. via the tail vein. Six days later, mice were divided into four groups (groups 1 to 4) of seven mice each. The experimental conditions and treatment schedule were identical to those used for the
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H1299-SCID/Beige lung tumor model. The effect of delivering DOTAP:Chol-*p53* DNA:liposome complex on survival was calculated using the Kaplan-Meier survival estimation and the Wilcoxon rank test.

5 **Statistical Analysis:** The statistical significance of the experimental results was calculated using Student's *t*-test for tumor measurements, Mann-Whitney rank-sum test for lung metastases, Wilcoxon log rank test and Kaplan-Meier survival test for animal survival experiments.

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EXAMPLE 9

IN VIVO TRANSFECTION IN NORMAL LUNG, PRIMARY TUMOR XENOGRAFTS AND EXPERIMENTAL METASTATIC LUNG TUMORS

The ability of extruded DOTAP:Chol liposomes to effectively transfect and 15 deliver plasmid DNA into normal lung, subcutaneous lung tumor xenografts, and experimental metastatic lung tumors was determined using expression plasmids encoding the bacterial β -galactosidase (*Lac-Z*), human *p53*, or human *Fhit* protein. 48 hours following a single i.v. tail vein injection of DOTAP:Chol liposomes complexed with *Lac-Z* plasmid DNA or human *p53* plasmid DNA (50 μ g of DNA) that were 300-325 nm in 20 size, alveolar epithelial cells (type II pneumocytes) and endothelial cells in the lung were largely observed to produce β -galactosidase or *p53* protein. Few alveolar macrophages expressed the transgene. In contrast, no gene expression was observed in animals injected with naked *p53* plasmid DNA (50 μ g).

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To determine whether gene expression could also be achieved in solid primary tumors, human H1299 lung tumors were established subcutaneously in nude mice. Forty-eight hours following a single intratumoral injection of DOTAP:Chol-*LacZ* DNA:liposome complex, 25% of the tumor cells produced β -galactosidase, as shown by histochemical staining. In contrast, no β -galactosidase production was observed in the

untreated control tumors. Intratumoral injection of DOTAP:Chol liposomes complexed to *FHIT* (50 µg of DNA) resulted in production of Fhit protein, as determined by western blot analysis. Tumor-bearing animals injected with DOTAP:Chol liposomes complexed to *CAT* DNA served as controls. Similarly, p53 protein expression was observed by 5 western blot analysis.

The ability to transfect experimental human A549 lung metastatic tumors established in nude mice was also evaluated. A single tail vein injection of DOTAP:Chol liposomes complexed to *Lac-Z* or *FHIT* plasmid DNA (50 µg) into lung tumor-bearing 10 mice resulted in 10% of the tumor cells producing β-galactosidase and Fhit protein at 48 hours.

EXAMPLE 10

IN VIVO EVALUATION OF LOCAL TUMOR GROWTH SUPPRESSION BY P53 AND FHIT

15 The ability of the DOTAP:Chol-*p53* DNA:liposome complex to suppress the growth of *p53* gene-null H1299 human lung subcutaneous tumors in *nu/nu* mice was assessed. Tumor-bearing mice were divided into three groups: one receiving no treatment, one treatment with naked *p53* plasmid DNA, and one treatment with the 20 DOTAP:Chol-*p53* DNA liposome complex daily for a total of six doses (100 µg/dose). Tumor growth was significantly inhibited ($p = 0.001$) in mice treated with the DOTAP:Chol-*p53* liposome complex (FIG. 1a) as compared with tumor growth in the no treatment and *p53* plasmid DNA control groups.

25 To further demonstrate the specific tumor-suppressive effects of the *p53* gene delivered by DOTAP:Chol liposomes, in a separate set of experiments, subcutaneous H1299 tumor-bearing animals were divided into three groups, one receiving no treatment, one treatment with DOTAP:Chol liposome complexed to irrelevant plasmid DNA (*pAd*) and one treatment with the DOTAP:Chol-*p53* DNA complex daily for a total of six doses

(100 µg/dose). Significant inhibition of tumor growth was not observed in animals that were either not treated or treated with the DOTAP:Chol liposome complexed to irrelevant plasmid DNA (FIG. 1b). In contrast, animals treated with the DOTAP:Chol-*p53* DNA:liposome complex showed significant tumor inhibition ($p = 0.01$).

5

Further evidence that the observed therapeutic effect was due to *p53* gene expression was obtained by removing subcutaneous tumors 48 hours after injection and analyzing them for *p53* gene expression by immunohistochemistry. *p53* gene expression was seen in 39% of tumor cells in animals receiving the DOTAP:Chol-*p53* liposome complex, (FIG. 1c) ($p = 0.001$), as compared with the percentage in control tumors that were either not treated or treated with *p53* plasmid DNA. Analysis of apoptotic tumor cell death showed that 32% of the tumor cells in mice receiving the DOTAP:Chol-*p53* liposome complex were positive in TUNEL studies ($p = 0.001$) (FIG. 1d). Tumors from control mice that were either untreated or treated with *p53* plasmid DNA showed minimal apoptotic cell death (FIG. 1d).

The therapeutic effect of the *FHIT* tumor suppressor gene on H1299 and A549 subcutaneous tumors in nude mice was similarly evaluated. Mice bearing each tumor cell type (H1299 and A549) were divided into four groups: one receiving no treatment, one treatment with naked *FHIT* plasmid DNA, one treatment with the DOTAP:Chol-*CAT* DNA liposome complex, and one treatment with the DOTAP:Chol-*FHIT* DNA liposome complex daily for a total of six doses (100 µg/dose). A significant growth inhibition of H1299 tumors ($p = 0.02$) (FIG. 2l) and A549 tumors ($p = 0.001$) (FIG. 2m) was observed in mice treated with the DOTAP:Chol-*FHIT* DNA liposome complex compared with the tumor growth in the three control groups for each tumor type.

EXAMPLE 11

IN VIVO EVALUATION OF P53 AND FHIT PLASMID DNA COMPLEXED TO DOTAP:CHOL LIPOSOMES FOR TREATMENT OF EXPERIMENTAL LUNG METASTASES

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The effectiveness of extruded DOTAP:Chol liposomes, over non-extruded DOTAP:Chol liposomes, and another conventional liposome formulation (DOTAP:DOPE) was compared in a therapeutic xenograft model of human lung metastases using the H1299 and A549 human lung cancer cells. There was a significant reduction ($p < 0.001$) in the number of metastases in the lungs from *p53* gene-null H1299 lung tumor-bearing SCID/Beige mice receiving the extruded DOTAP:Chol-*p53* DNA:liposome complex (FIG. 2a) as compared with the number in mice receiving no treatment, *p53* plasmid DNA, the extruded DOTAP:Chol-CAT DNA:liposome complex, the DOTAP:DOPE-*p53* DNA:liposome complex, or the nonextruded DOTAP:Chol-*p53* DNA:liposome complex. In addition, metastatic tumor growth was inhibited in animals treated with the extruded DOTAP:Chol-CAT DNA:liposome complex and the DOTAP:DOPE-*p53* DNA:liposome complex compared with tumor growth in mice receiving no treatment, *p53* plasmid DNA, or nonextruded DOTAP:Chol *p53* DNA complex. However, the tumor inhibitory effects were minimal.

15

To determine if the observed *p53* gene-mediated tumor inhibitory effects were restricted to *p53* gene mutated or null tumors, the effects of the DOTAP:Chol-*p53* DNA:liposome complex were studied in A549 tumor cells, which are homozygous for the wild-type *p53* gene and form lung metastases following tail-vein injection in *nu/nu* mice. 20 A significant reduction ($p = 0.001$) in the number of metastases was observed in mice treated with the extruded DOTAP:Chol-*p53* DNA:liposome complex compared with the number of metastases in control mice that were either not treated or treated with the DOTAP:Chol-CAT DNA:liposome complex (FIG. 2b), thus ruling out the possibility that the *p53* gene mediated inhibition was limited to *p53* gene mutated or null tumors. No 25 significant difference was observed in the number of metastases between the two control groups.

The ability of the *FHIT* tumor suppressor gene to inhibit lung metastases formed by A549 tumor cells in nude mice was also evaluated. A significant reduction ($p = 30 0.007$) in the number of tumor metastases was observed in animals treated with the

DOTAP:Chol-*FHIT* DNA:liposome complex (FIG. 2c). In contrast, control animals that were not treated, treated with *FHIT* plasmid DNA, or treated with the DOTAP:Chol-*CAT* DNA:liposome complex showed no significant reduction in the number of tumor metastases.

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EXAMPLE 12

TREATMENT OF LUNG TUMORS WITH DOTAP:CHOL-P53 DNA:LIPOSOME COMPLEX

RESULTS IN APOPTOTIC TUMOR CELL DEATH

To determine the fate of tumor cells after treatment with the DOTAP:Chol-*p53* DNA:liposome complex, A549 lung tumors from *nu/nu* mice were analyzed histologically, and apoptotic cell death was assessed by TUNEL staining. Histopathological examination of the lung sections from mice treated with the DOTAP:Chol-*p53* DNA:liposome complex showed the presence of very few metastases that were small and contained only a few viable tumor cells. In addition, the number of tumor metastases in the lungs of these animals was significantly less than that in control animals. Further, apoptotic cell death had occurred in these tumors, as demonstrated by TUNEL staining. In contrast, lungs from control mice that received no treatment showed several large tumors with numerous mitoses and no evidence of apoptotic cell death.

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EXAMPLE 13

ANIMAL SURVIVAL IN A MODEL OF DISSEMINATED HUMAN LUNG CANCER

To determine the efficacy of the systemically administered extruded liposomal tumor suppressor gene complex, survival experiments were performed using the H1299 and A549 lung metastasis tumor models. H1299 lung tumor-bearing SCID/Beige mice were divided into four groups, as follows: no treatment (Group 1), *p53* plasmid DNA (Group 2), DOTAP:Chol-*CAT* DNA:liposome complex (Group 3), and DOTAP:Chol-*p53* DNA:liposome complex (Group 4). Animals were injected daily via tail vein for a total of six doses (50 µg/dose) and monitored daily following the last treatment to assess morbidity and mortality. Mice from Groups 1, 2, and 3 died from the tumor burden between 30 and 60 days after tumor cell injection (median survival time: 38 days in

Group 1, 40 days in Group 2, and 41 days in Group 3). In contrast, mice treated with the DOTAP:Chol-*p53* DNA:liposome complex (Group 4) survived for significantly longer period (median survival: 76 days; $p = 0.001$). In fact, 33% of mice from Group 4 were still alive on day 150 at the end of the experiment (FIG. 3a). Postmortem analysis of 5 organs from animals in all four groups showed no treatment related toxicity. However, dissemination of lung tumors to multiple organs and sites that included the cervical lymph node, intestine, mesenteric lymph nodes, liver, kidney, spleen, pancreas, adrenal, ovaries, uterus, peritoneal cavity with ascites, and bone was revealed.

10 Survival in A549 lung tumor-bearing *nu/nu* mice was also evaluated. Following tumor cell injection, animals were divided into four groups, as follows: no treatment (Group 1), *p53* plasmid DNA (Group 2), DOTAP:Chol-CAT DNA:liposome complex (Group 3), and DOTAP:Chol-*p53* DNA:liposome complex (Group 4). The treatment schedule was identical to that followed in the H1299/SCID/Beige model. Mice in Group 15 4, which received the DOTAP:Chol-*p53* DNA:liposome complex, showed prolonged survival (median survival, 96 days; $p = 0.04$) as compared with mice in the three control groups (median survival: 50 days in Group 1, 49 days in Group 2, and 52 days in Group 3) (FIG. 3b). Histopathological analysis of various organs revealed extensive tumor spread in the lungs of all four groups of animals, but dissemination to other organs was 20 not observed in animals in any of the four groups. In addition, treatment associated toxicity was not observed in animals from any of the four groups.

25 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the 30 method described herein without departing from the concept, spirit and scope of the

invention. More specifically, it will be apparent that certain agents, which are both chemically and physiologically related, may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope
5 and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein
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WHAT IS CLAIMED IS:

1. A pharmaceutically acceptable lipid formulation comprising DOTAP and at least one cholesterol or cholesterol derivative or cholesterol mixture, in combination with a
 - (a) nucleic acid antisense or ribozyme molecule that inhibits the expression of a growth-promoting polypeptide; or
 - (b) nucleic acid under the control of a promoter and encoding an (1) anti-proliferative polypeptide or (2) an antisense molecule or a ribozyme that inhibits the expression of a growth-promoting protein.
2. The formulation of claim 1, comprising DOTAP in a concentration ranging from about 1 to about 8 mM.
3. The formulation of claim 2, comprising DOTAP in a concentration ranging from about 2 to about 7 mM.
- 20 4. The formulation of claim 3, comprising DOTAP in a concentration ranging from about 3 to about 6 mM.
5. The formulation of claim 4, comprising DOTAP in a concentration of about 4 to about 5 mM.
- 25 6. The formulation of claim 1, comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration ranging from about 1 to about 8 mM.
7. The formulation of claim 6, comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration ranging from about 2 to about 7 mM.

8. The formulation of claim 7, comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration ranging from about 3 to about 6 mM.
- 5 9. The formulation of claim 8, comprising the cholesterol or cholesterol derivative or cholesterol mixture in a concentration of about 4 to about 5 mM.
10. The formulation of claim 1, wherein the DOTAP and cholesterol or cholesterol derivative or cholesterol mixture are included in a molar ratio from about 3:1 to about 1:3.
11. The formulation of claim 10, wherein the DOTAP and cholesterol or cholesterol derivative or cholesterol mixture are included in a molar ratio of about 1:1.
- 15 12. The formulation of claim 1, wherein the formulation is extruded.
13. The formulation of claim 1, wherein the anti-proliferative polypeptide is a tumor suppressor protein..
- 20 14. The formulation of claim 13, wherein the tumor suppressor polypeptide is Rb, p53, p14, p15, p16, p19, INK4c, p21, p27, p73, a p16-p27 fusion, a p21-p27 fusion, p56^{RB}, E2F-1, NOEY2, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC, PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2, mda-7, DBCCR-1 or WT-I.
- 25 15. The formulation of claim 14, wherein the tumor suppressor polypeptide is p53.
16. The formulation of claim 1, wherein the growth promoting polypeptide is a growth factor receptor.

30

17. The formulation of claim 1, wherein the anti-proliferative polypeptide is a single chain antibody, a decoy, or a dominant negative directed to a growth factor receptor.

18. The formulation of claim 17, wherein the growth factor receptor is FMS,
5 ERBB/HER, ERBB-2/NEU/HER-2, ERBA, TGF- β receptor, PDGF receptor, MET, KIT or TRK.

19. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits
the expression of a growth factor receptor.

10

20. The formulation of claim 19, wherein the growth factor receptor is FMS,
ERBB/HER, ERBB-2/NEU/HER-2, ERBA, TGF- β receptor, PDGF receptor, MET, KIT or TRK.

15

21. The formulation of claim 1, wherein the anti-proliferative polypeptide is a single chain antibody, a decoy, or a dominant negative directed to a signal transducing protein.

22. The formulation of claim 21, wherein the signal transducing protein is SRC, ABL,
RAS, AKT/PKB, RSK-1, RSK-2, RSK-3, RSK-B, PRAD, LCK, or ATM.

20

23. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits
the expression of a signal transducing protein.

25

24. The formulation of claim 23, wherein the signal transducing protein is SRC, ABL,
RAS, AKT/PKB, RSK-1, RSK-2, RSK-3, RSK-B, PRAD, LCK, or ATM.

25. The formulation of claim 1, wherein the anti-proliferative polypeptide is a single chain antibody, a decoy, or a dominant negative directed to a transcription factor.

26. The formulation of claim 25, wherein the transcription factor is JUN, FOS, MYC, BRCA1, BRCA2, ERBA, ETS, EVII, MYB, HMGI-C, HMGG/LIM, SKI, VHL, WT1, CEBP- α , NF κ B, I κ B, GL1, or REL.

5 25. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits
the expression of a transcription factor protein.

27. The formulation of claim 25, wherein the transcription factor protein is JUN,
FOS, MYC, BRCA1, BRCA2, ERBA, ETS, EVII, MYB, HMGI-C, HMGG/LIM, SKI,
10 VHL, WT1, CEBP- α , NF κ B, I κ B, GL1, or REL.

28. The formulation of claim 1, wherein the anti-proliferative polypeptide is a single
chain antibody, a decoy, or a dominant negative directed to a growth factor.

15 29. The formulation of claim 28, wherein the growth factor is SIS, HST, INT-1/WT1,
or INT-2.

-30. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits
the expression of a growth factor.

20 31. The formulation of claim 30, wherein the growth factor is SIS, HST, INT-1/WT1,
or INT-2 .

25 32. The formulation of claim 1, wherein the anti-proliferative polypeptide is an
apoptotic factor protein.

33. The formulation of claim 32, wherein the apoptotic factor protein is Bax, Bak,
Bim, Bik, Bcl-xL, Bcl-xS, Bid, Bad, Bcl-2, Harakiri, or an ICE protease.

34. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits the expression of an apoptotic factor protein.
35. The method of claim 34, wherein the apoptotic factor protein is Bax, Bak, Bim, Bik, Bcl-xL, Bcl-xS, Bid, Bad, Bcl-2, Harakiri, or an ICE protease.
36. The formulation of claim 1, wherein the anti-proliferative polypeptide is a single chain antibody, a decoy, or a dominant negative directed to a tumor-associated protein.
- 10 37. The formulation of claim 36, wherein the tumor-associated protein is CEA, mucin, MAGE, or GAGE.
38. The formulation of claim 1, wherein the antisense molecule or ribozyme inhibits the expression of a tumor-associated protein.
- 15 39. The formulation of claim 38, wherein the tumor-associated protein is CEA, mucin, MAGE, or GAGE.
40. The formulation of claim 1, further comprising a targeting moiety.
- 20 41. The formulation of claim 40, wherein the targeting moiety is a peptide, a ligand, or an antibody.
42. The formulation of claim 41, wherein the targeting moiety comprises a peptide that includes an RGD sequence.
- 25 43. The formulation of claim 42, wherein the peptide includes an RGDFV sequence.
44. The formulation of claim 43, wherein the peptide is from 3 to 30 amino acids in length.

45. The formulation of claim 44, wherein the peptide is from 3 to 20 amino acids in length.
- 5 46. The formulation of claim 45, wherein the peptide is from 4 to 10 amino acids in length.
47. The formulation of claim 43, wherein the peptide is a cyclic peptide.
- 10 48. The formulation of claim 47, wherein the cyclic peptide is 5 amino acids in length.
49. The formulation of claim 41, wherein the targeting moiety comprises a ligand that is a substrate for a cell surface protein.
- 15 50. The formulation of claim 49, wherein the cell surface protein is an integrin, proteoglycan, glycoprotein, receptor, or transporter.
51. The formulation of claim 41, wherein the targeting moiety comprises an antibody that binds to a cell surface protein.
- 20 52. The formulation of claim 51, wherein the antibody is an antibody to the Her-1 receptor.
- 25 53. The formulation of claim 1, wherein the promoter is CMV IE, CEA, VEGF, AFP, lung surfactant promoter, dectin-1, dectin-2, human CD11c, F4/80, SM22, CEA, tyrosinase, tet-inducible or tet-repressible, RSV, MLP, or MHC class II promoter.

54. A method of treating a hyperproliferative disorder comprising administering an effective amount of the formulation of claim 1 to a patient in need of anti-proliferative therapy.

5 55. The method of claim 54, wherein the hyperproliferative disease is cancer.

56. The method of claim 55, wherein the cancer is lung, head and neck, pancreatic, prostate, renal, bone, testicular, breast, cervical, gastrointestinal, lymphoma, brain, breast, ovarian, leukemia, myeloma, colorectal, esophageal, skin, thyroid, liver, or bladder
10 cancer.

57. The method of claim 54, wherein the hyperproliferative disease is rheumatoid arthritis, inflammatory bowel disease, osteo-arthritis, adenoma, leiomyoma, lipoma, hemangioma, fibroma, restenosis, pre-neoplastic lesions, vascular occlusion, or psoriasis.

15

58. The method of claim 54, wherein the hyperproliferative disorder is vascular occlusion.

59. The method of claim 58, wherein the hyperproliferative disorder is restenosis.

20

60. The method of claim 55, wherein the formulation is administered to the patient by intratumoral injection, intratracheal injection, intravenous injection, intraperitoneal injection, intravesical instillation, intra-arterial infusion, intrapericardial injection, intramuscular injection, topical application, aerosolization, mucosal exposure, orally, or
25 lavage.

61. The method of claim 54, wherein the formulation is administered more than one time.

62. The method of claim 60, wherein the formulation is administered to the patient by intratumoral injection.
63. The method of claim 60, wherein the formulation is administered intravenously to
5 the patient.
64. The method of claim 55, wherein the cancer comprises a tumor.
65. The method of claim 64, wherein the formulation is administered to a tumor bed
10 prior to or after resection of the tumor.
66. The method of claim 65, wherein the formulation is administered to the tumor bed both prior to and after tumor resection.
- 15 67. The method of claim 54, wherein the formulation is administered to the patient before chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.
68. The method of claim 54, wherein the formulation is administered during
chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.
20
69. The method of claim 54, wherein the formulation is administered after chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.
70. The method of claim 69, wherein the formulation is administered less than 72
25 hours prior to chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.
71. The method of claim 70, wherein the formulation is administered less than 24 hours prior to chemotherapy, surgery, immunotherapy, hormonal therapy, or
30 radiotherapy.

72. The method of claim 71, wherein the formulation is administered less than 72 hours after chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.
- 5 73. The method of claim 72, wherein the formulation is administered less than 24 hours after chemotherapy, surgery, immunotherapy, hormonal therapy, or radiotherapy.

10

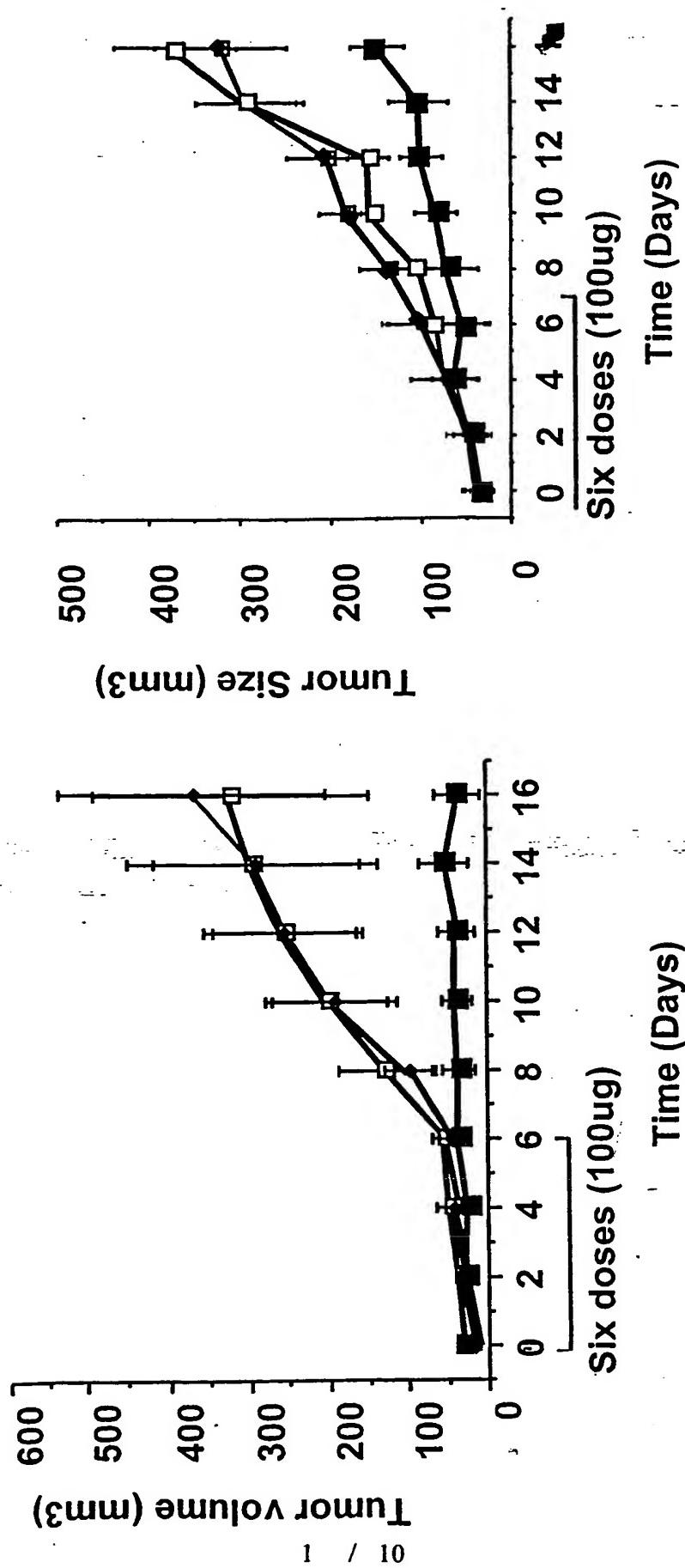


FIG. 1A

FIG. 1B

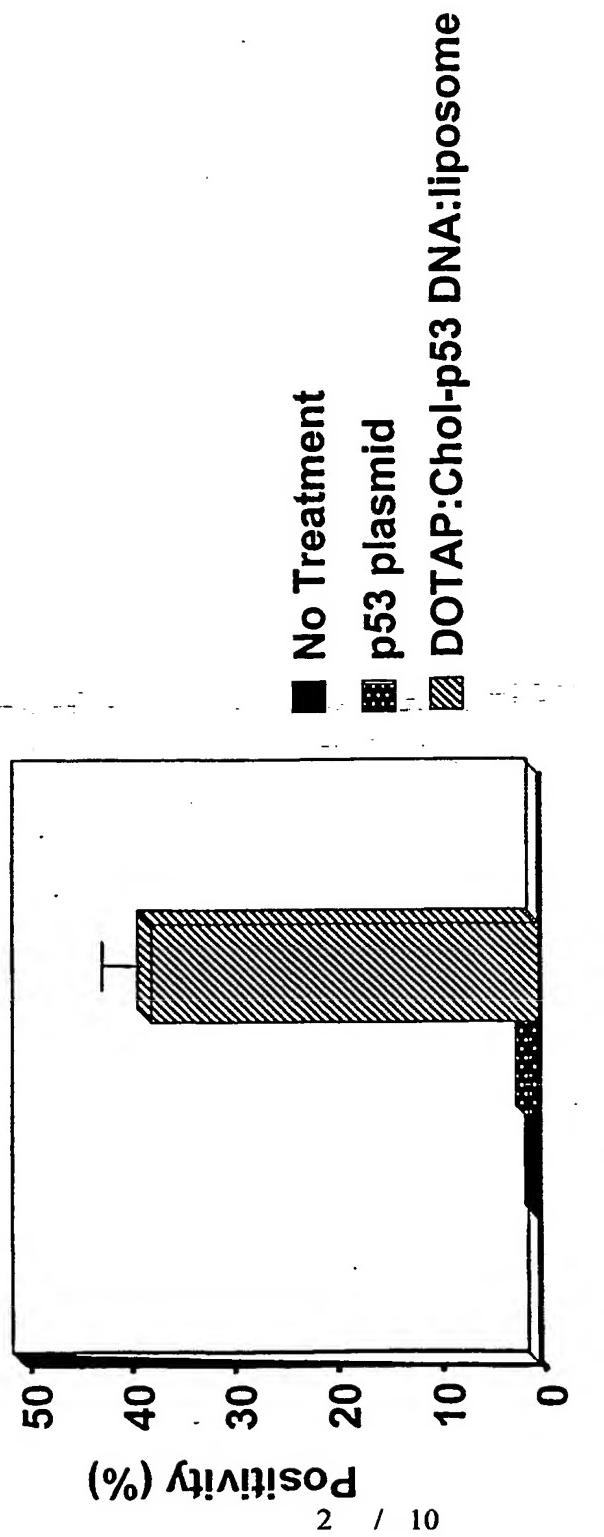


FIG. 1C

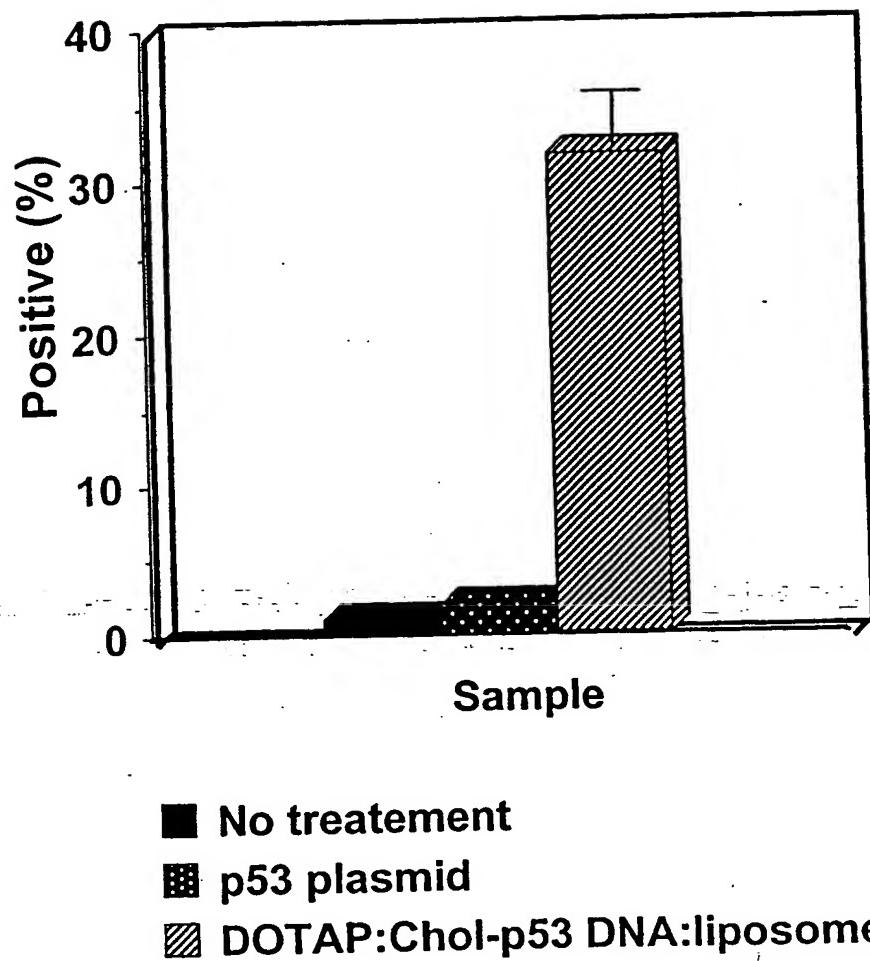


FIG. 1D

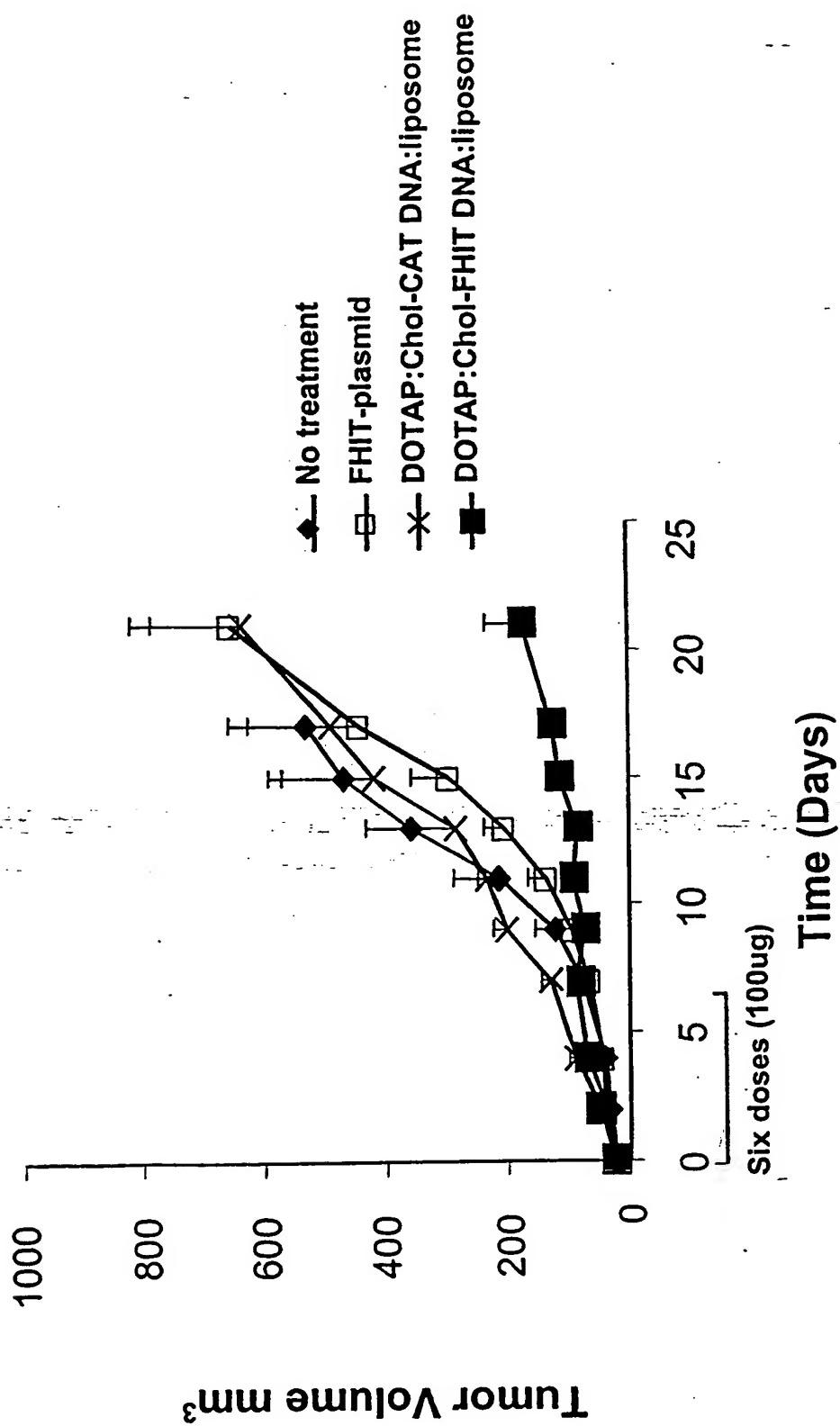


FIG. 1E

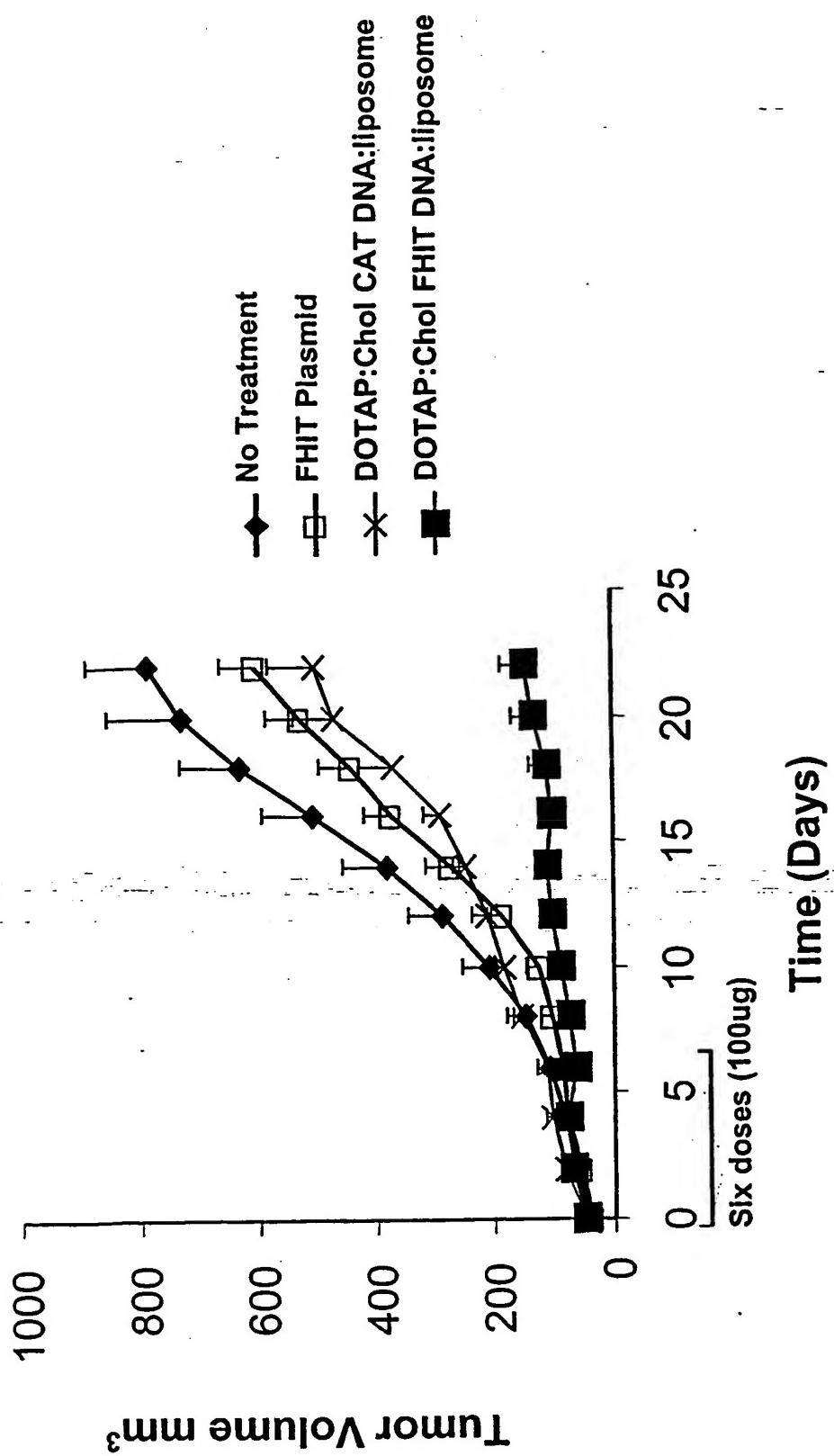


FIG. 1F

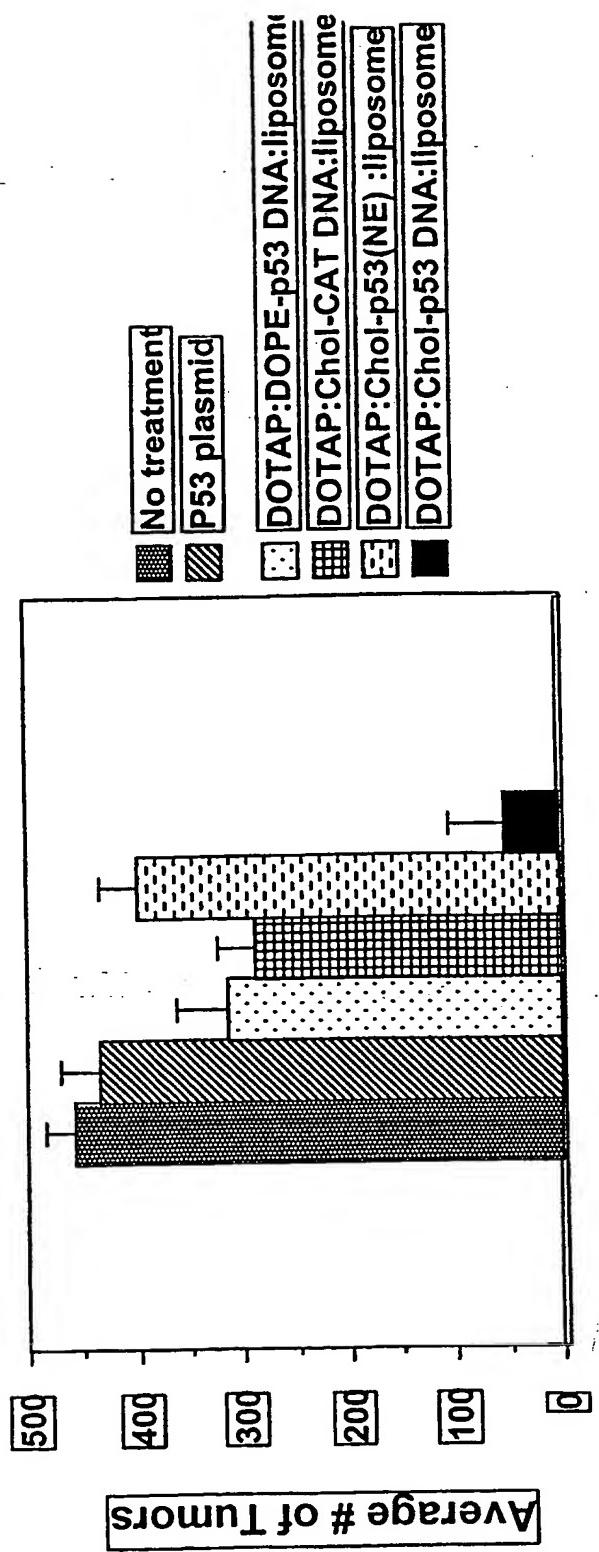


FIG. 2A

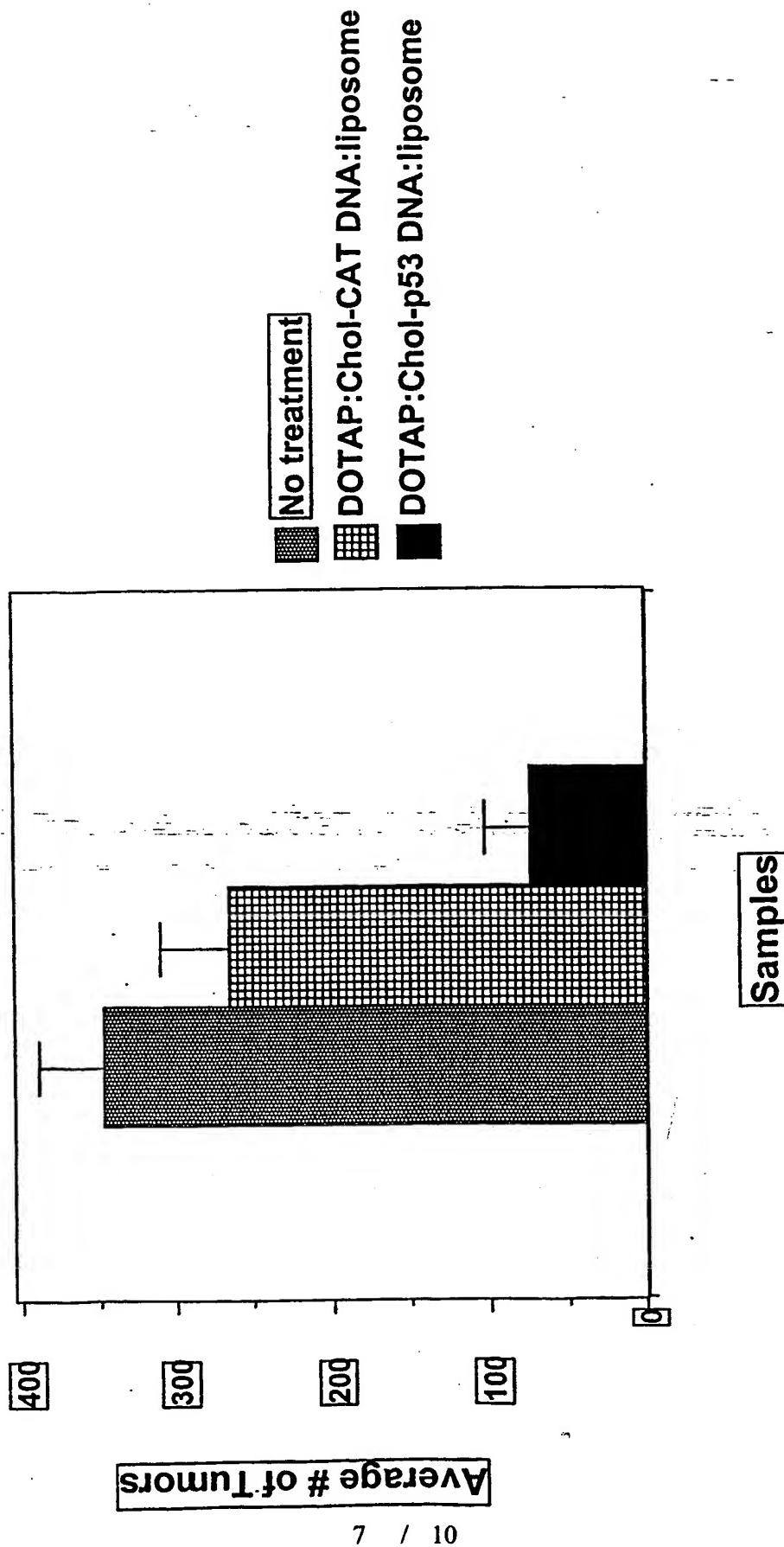


FIG. 2B

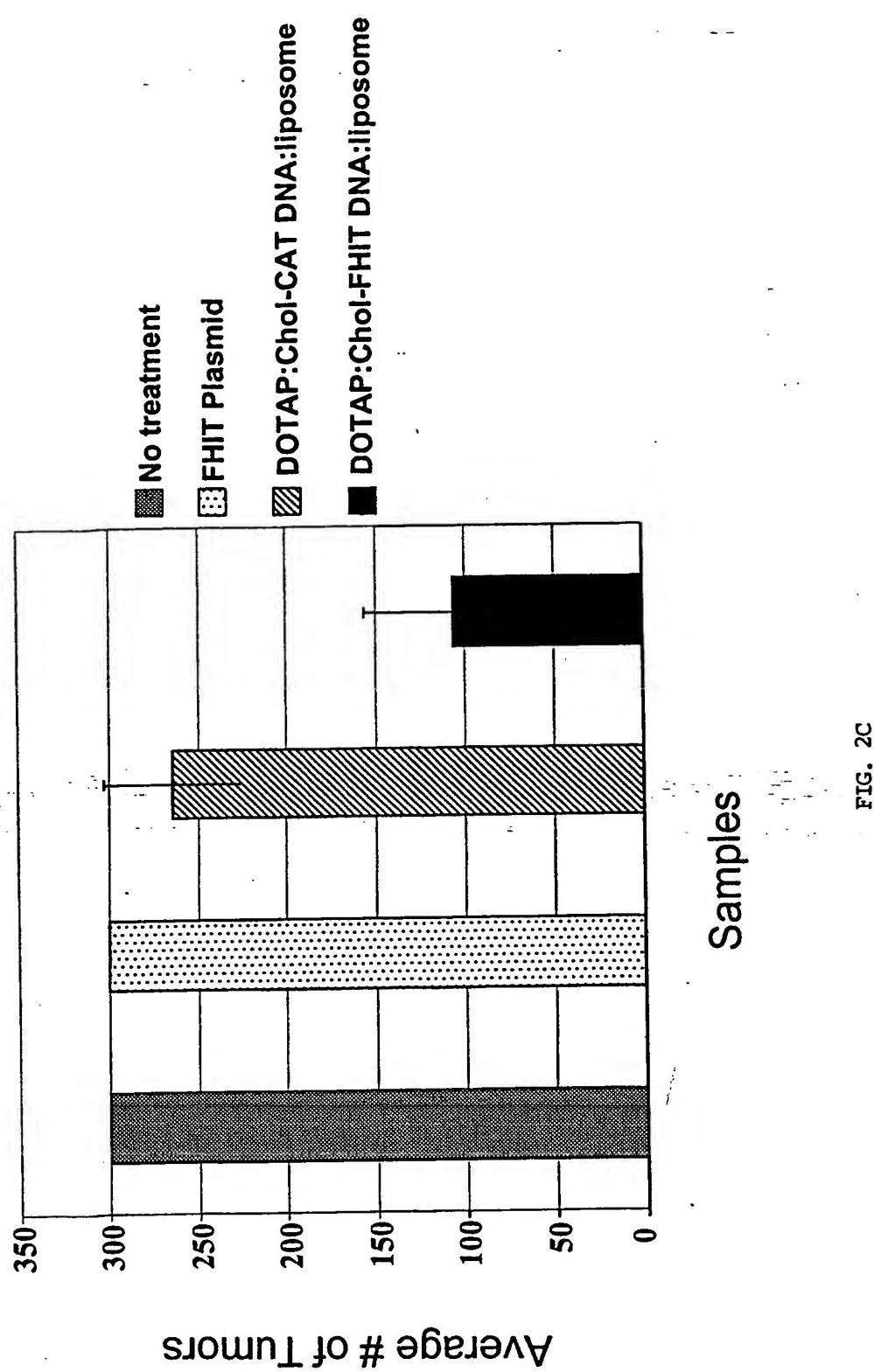


FIG. 2C

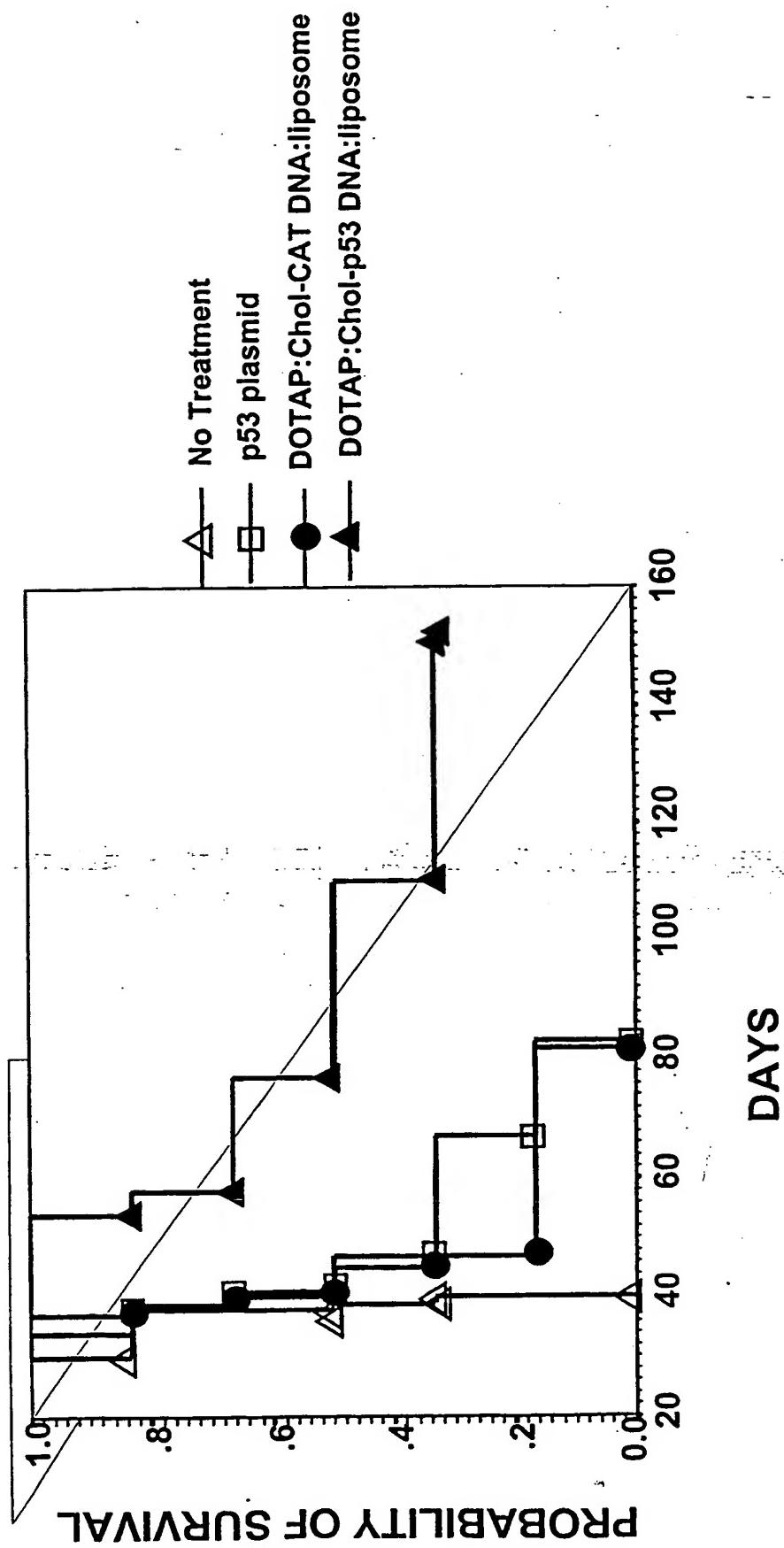


FIG. 3A

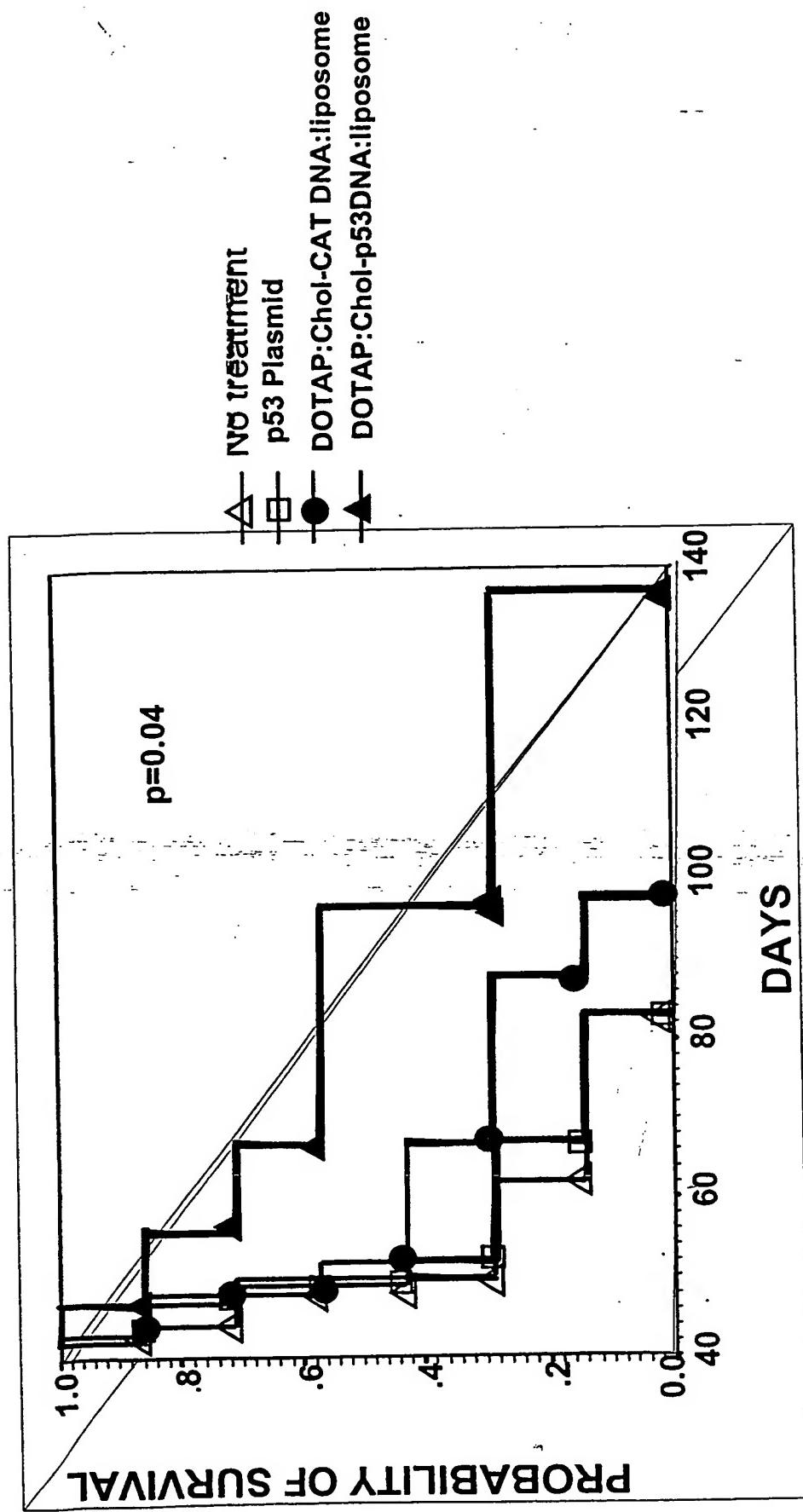


FIG. 3B

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Tokyo 158-0082 (JP). WILSON, Deborah [US/US];
11022 Silkwood, Houston, TX 77031 (US).

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(74) Agent: SHISHIMA, Gina, N.: Fulbright & Jaworski,
L.L.P., Suite 2400, 600 Congress Avenue, Austin, TX
78701 (US).

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(71) Applicants (*for all designated States except US*): INTRO-
GEN THERAPEUTICS, INC. [US/US]; Suite 1850, 301
Congress Avenue, Austin, TX 78701 (US). BOARD OF
REGENTS, THE UNIVERSITY OF TEXAS SYSTEM
[US/US]; 201 West 7th St., Austin, TX 78701 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): RAMESH, Ra-
jagopal [IN/US]; 5400 Braesvalley No. 96, Houston,
TX 77096 (US). ROTH, Jack, A. [US/US]; 6516
Brompton Road, Houston, TX 77005 (US). SAEKI,
Tomoyuki [JP/JP]; 6-33-18-A Todoroki, Setagaya-ku,

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(54) Title: METHODS AND COMPOSITIONS FOR NON-VIRAL GENE THERAPY FOR TREATMENT OF HYPERPROLIF-
ERATIVE DISEASES

(57) Abstract: The present invention relates to non-viral gene therapy methods and compositions for treatment of hyperproliferative disease in humans. More specifically, the invention is directed, in one embodiment, to lipid formulations which form stable liposome structures, capable of efficient *in vivo* nucleic acid transfer. In other embodiments, methods and compositions are directed to liposome transfer of anti-proliferative nucleic acids, wherein the transfer of the nucleic acids is cell specific *via* cell specific targeting moieties. The present invention, thus provides non-viral, liposome compositions and methods of gene transfer particularly useful for targeting and treating hyperproliferative disease.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K48/00 A61K31/7088 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 98 07408 A (PAVLAKIS GEORGE N ;SMYTH TEMPLETON NANCY (US); US HEALTH (US)) 26 February 1998 (1998-02-26) cited in the application abstract page 3, paragraph 8 -page 9, paragraph 3 page 17, paragraph 4 -page 22, paragraph 2 page 14, paragraph 1 - paragraph 3</p> <hr/> <p>WO 93 24640 A (UNIV CALIFORNIA ;DEBS ROBERT J (US); ZHU NING (US)) 9 December 1993 (1993-12-09) abstract page 24, line 20 - line 26 page 29, line 5 - line 35 page 32, line 29 - line 35 page 53; claims 1,5,9; example 26</p> <hr/> <p>-/-</p>	1-74
X		1-74

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Niemann, F

INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 98 20857 A (UNIV CALIFORNIA) 22 May 1998 (1998-05-22)</p> <p>abstract page 14, line 10 - line 11 page 18, line 21 -page 22, line 29 page 32, line 6 - line 29</p> <p>-----</p>	1-74
A	<p>TEMPLETON NANCY SMYTH ET AL: "Improved DNA: Liposome complexes for increased systemic delivery and gene expression." NATURE BIOTECHNOLOGY, vol. 15, no. 7, 1997, pages 647-652, XP002154098</p> <p>ISSN: 1087-0156 the whole document</p> <p>-----</p>	
P,X	<p>RAMESH RAJAGOPAL ET AL: "Inhibition of primary and disseminated human lung cancers by systemic delivery of p53 tumor suppressor gene using an improved liposome." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL, no. 41. March 2000 (2000-03), page 603 XP002154099</p> <p>91st Annual Meeting of the American Association for Cancer Research.; San Francisco, California, USA; April 01-05, 2000, March, 2000</p> <p>ISSN: 0197-016X the whole document</p> <p>-----</p>	1-74

INTERNATIONAL SEARCH REPORT

Information on patent family members			Int'l. Application No PCT/US 00/14350
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WO 9324640	A 09-12-1993	AU 1912897 A AU 3467193 A AU 672412 B AU 4407593 A AU 4528493 A CA 2126101 A CA 2134773 A EP 0625207 A EP 0646178 A JP 7507450 T JP 7502510 T WO 9312240 A WO 9325673 A US 5827703 A US 6001644 A	26-06-1997 19-07-1993 03-10-1996 30-12-1993 04-01-1994 24-06-1993 09-12-1993 23-11-1994 05-04-1995 24-08-1995 16-03-1995 24-06-1993 23-12-1993 27-10-1998 14-12-1999
WO 9820857	A 22-05-1998	AU 7177998 A EP 0956001 A	03-06-1998 17-11-1999